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FRESHWATER CYANOBACTERIA (BLUE-GREEN ALGAE) TOXINS: ISOLATION AND CHARACTERIZATION

ANNUAL REPORT

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Freshwater cyanobacteria (blue-green algae) are capable of producing several physiologically distinct toxins. These toxins are produced by strains of the bloom-forming species Anabaena flos-aquae, Microcystis aeruginosa, Aphanizomenon flos-aquae, Oscillatoria agardhii and Nodularia spumigena. Work carried out under this contract covered the following areas with these biotoxin-producing microorganisms: 1) Culture, using batch and continuous culture methods, selected neuro- and hepatotoxin-producing strains of freshwater cyanobacteria. 2) Extract and purify the toxins using organic extraction followed by gel and ion-exchange column filtration (both standard and high performance liquid chromatography (HPLC)). 3) Provide USAMRIID with selected purified hepatotoxic peptides and neurotoxic alkaloids. 4) Selected studies on optimization of culture conditions for toxic production and storage of toxic strains. 5) Continue collaborative studies in other areas of the U.S. and the world to isolate and compare freshwater/marine cyanobacteria toxins so that common methods of detection/decontamination can be developed.

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SUMMARY CONTENTS

This annual report covers work completed and in progress on "Freshwater Cyanobacteria (blue-green algae) Toxins: Isolation and Characterization". The first part of the report updates review material on toxins of freshwater cyanobacteria. The second part details studies covered under this contract as described in the contract workscope. The workscope areas include: 1) Development of culture methods of neuro- and hepatotoxin producing strains of freshwater cyanobacteria. This work has centered on implementation of fermenter systems designed for semi-continuous harvesting of algal cells, in addition to optimization of culture conditions for control of toxin production. 2) Extraction, purification and analysis of neurotoxins and hepatotoxins. This work has centered on purification and analysis of cyclic peptide toxins of Microcystis aeruginosa and Nodularia spumigena, and the neurotoxin ANTX-A(S) from Anabaena flos-aquae. 3) Toxicology work has involved the isolation, purification and enzyme kinetics of the anti-cholinesterase compound called anatoxin-a(s). 4) Collaborative studies to investigate new occurrences of toxic blue-green algae and to isolate, culture, and examine new toxic species. This work has resulted in the examination and isolation of new toxic isolates of Microcystis aeruginosa form Wisconsin and Illinois, Oscillatoria agardhii from Norway, Nodularia spumigena from New Zealand, Nostoc sp. and Nodularia spumigena from Finland and Microcystis aeruginosa from the Peopies Republic of China.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of The Institute of Laboratory Animal Resources, National Research Council. (DHEW Publication No. (NIH) 86-23, Revised 1985.)

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A. REVIEW UPDATE OF CYANOBACTERIA TOXINS

1. INTRODUCTION

Reports of toxic algae in the freshwater environment are almost exclusively caused by strains of species that are members of the division Cyanophyta, commonly called blue-green algae or cyanobacteria. Although cyanobacteria are found in almost any environment ranging from hot springs to Antarctic soils, known toxic members are mostly planktonic. Published accounts of field poisonings by cyanobacteria are known since the late 19th century (Francis, 1878). These reports describe sickness and death of livestock, pets, and wildlife following ingestion of water containing toxic algae cells or the toxin released by the aging cells. Recent reviews of these poisonings and the toxins of freshwater cyanobacteria are given by Carmichael (1981, 1986, 1988), Codd and Bell (1985) and Gorham and Carmichael (1988).

While about 12 genera have been implicated in cyanobacteria poisonings only toxins from Anapaena, Aphanizomenon, Microcystis, Nodularia, and Oscillatoria have been isolated, at least partially chemically defined and the toxins studied for their mode of action. In addition to the acute lethal toxins, some cyanobacteria produce potent cytotoxins. These secondary chemicals are not considered in this chapter but the reader is referred to papers by Barchi et al. (1983, 1984); Carmichael (1988); Moore et al. (1984, 1986); Mason et al. (1982) and Gleason and Paulson (1984) for further discussion of these compounds. These cytotoxins are also listed in Table 2.

Economic losses related to freshwater cyanobacterial toxins are the result of contact with or consumption of water containing toxin and/or toxic cells. These toxins are water-soluble and temperature-stable. They are either released by the cyanobacterial cell or loosely bound so that changes in cell permeability or age allow their release into the environment. Lethal and sublethal amounts of these toxins become available to animals during periods of heavy cell growth, termed "waterblooms," especially when the waterbloom accumulates on the surface, inshore, where animals are watering. Waterblooms can occur wherever proper conditions for growth, including irradiance, temperature, neutral or alkaline conditions, and nutrients are found. The increasing eutrophication of water supplies from urban and agricultural sources, which raises mineral nutrient levels, has increased the occurrence and intensity of these annual blooms. It should be noted that although there are several bloom-forming genera of cyanobacteria those that occur most often are also those that can produce toxins. Known occurrences of toxic cyanobacteria in water supplies (Table 1), include Canada (four provinces, Europe (12 countries), United States (20 states), USSR (Ukraine), Australia, India, Bangladesh, South Africa, Israel, Japan, New Zealand, Argentina, Chile and the Peoples Republic of China (Skulberg, et al., 1984; Carmichael et al., 1985, Gorham and Carmichael, 1988). Not all blooms of a toxigenic species produce toxins, however,

and it is not possible to tell by microscopic examination of the cells whether they are toxic. Environmental conditions that favor bloom formation include (1) moderate to high levels of nutrients, especially phosphorus and nitrate or ammonia, (2) water temperatures between 15 and 30°C, and (3) a pH between 6 and 9 or higher (Skulberg et al., 1984). The economic impact from toxic freshwater cyanobacteria include the costs incurred from deaths of domestic animals; allergic and gastrointestinal problems after human contact with water blooms (including lost income from recreational areas); and increased expense for the detection and removal of taste, odor, and toxins (although no approved method yet exists for removal of toxins, activated carbon has been tried in certain areas). This section summarizes the neurotoxins and hepatotoxins of fresh and brackish water cyanobacteria. A summary of these compounds is given in Table 2.

Table 1. Known Occurrences of Toxic Cyanobacteria in Fresh or Marine Water (updated from Gorham and Carmichael, 1988)

ARGENTINA	INDIA
AUSTRALIA	ISRAEL
BANGLADESH	JAPAN
BERMUDA	NEW ZEALAND
BRAZIL	OKINAWA (MARINE)
CANADA	PEOPLES REPUBLIC OF CHINA
Alberta	SOUTH AFRICA
Manitoba	
Ontario	U.S.A.
Saskatchewan	
	California
EUROPE	Colorado
	Hawaii (marine)
Czechoslovakia	Idaho
Denmark	Illinois
East Germany	Iowa
Finland	Michigan
Great Britain	Minnesota
Hungary	Montana
Netherlands	Nevada
Norway	New Hampshire
Poland	New Mexico
Portugal	New York
Sweden	North Dakota
West Germany	Oregon
	Pennsylvania
	South Dakota
	Texas
	Washington
	Wisconsin
	U.S.S.R.
	Ukraine

World map showing areas (darkened) where toxic freshwater cyanobacteria have been found.

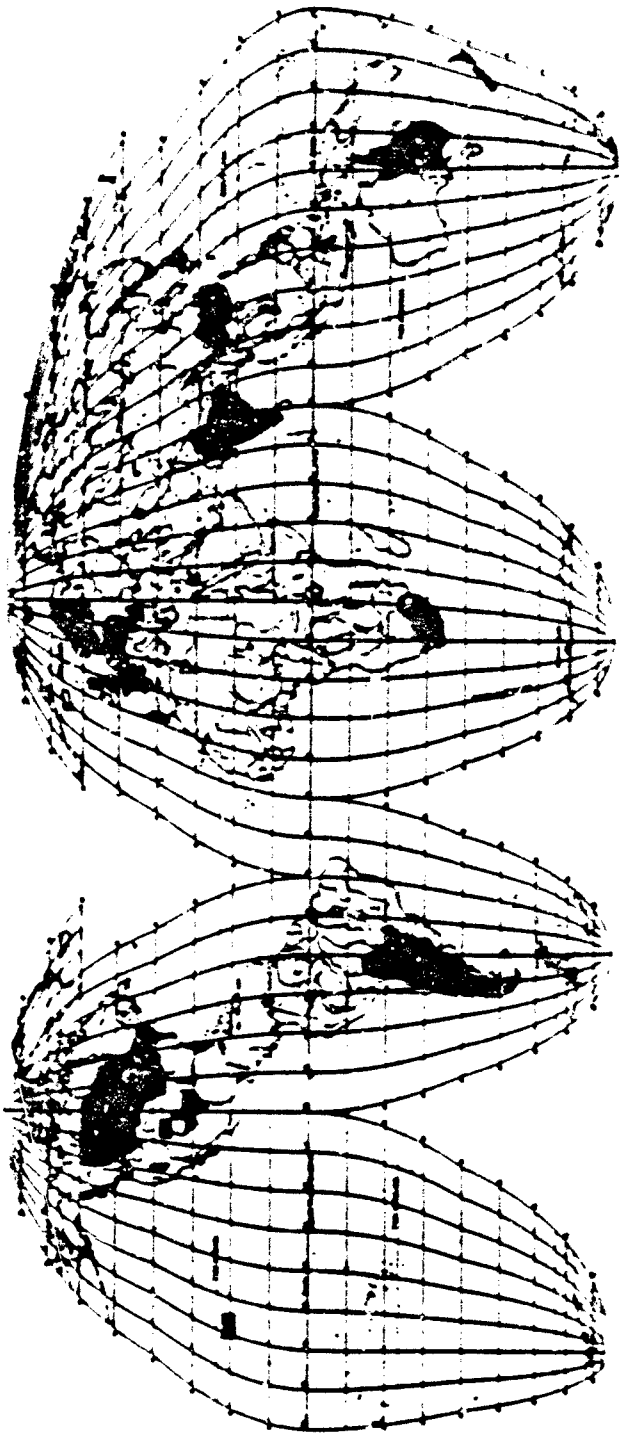


Table 2. Toxins of Freshwater Cyanobacteria

Species, strain, and source	Toxin term	Structure	IP, mouse
Neurotoxins			
Anabaena flos-aquae	Anatoxin-A	Secondary amine alkaloid, MW 165	200
Strain NRC-44-1 (Canada, Saskatchewan)			
Strain NRC-525-17 (Canada, Saskatchewan)	Anatoxin-A(S)	Unknown	50
Aphanizomenon flos-aquae	Aphanotoxin I (neosaxitoxin)	Purine alkaloid MW 315 (neoSTX) MW 299 (STX)	10
Strain NH-1 & NH-5 U.S., New Hampshire)	Aphantoxin II (saxitoxin)		
Hepatotoxins			
Anabaena flos-aquae	Microcystins[a]	Heptapeptides MW 994	50
Strain S-23-g-1 (Canada, Saskatchewan)			
Microcystis aeruginosa	Cyanoginosins[a]	Heptapeptides MW 909-1044	50
Strain WR-70 (South Africa, Transvaal)			
(Waterbloom, Australia, New South Wales)	Cyanoginosin	Heptapeptide MW 1035	50
(Waterbloom, U.S., Wisconsin)	Microcystin	Heptapeptide MW 994	50

Strain NRC-1(SS-17) (Canada, Ontario)	Microcystin	Heptapeptide MW 994	50
Strain 7820 (Scotland, Loch Baigaves)	Microcystin	Heptapeptide MW 994	50
(Waterbloom, Norway, Lake Akersvatn)	Microcystin	Heptapeptide MW 994	50
Microcystis aeruginosa Strain M-228 (Japan, Tokyo)	Microcystin	Heptapeptide MW 994 MW 1044	50
Microcystis aeruginosa	Cyanogenosin	Heptapeptide MW 1039	not reported
Microcystis viridis	Cyanoviridin	Heptapeptide MW 1039	not reported
Nodularia spumigena	Nodularin	Pentapeptide MW 824	30-50
Oscillatoria agardhii var. isothrix (Waterbloom, Norway, Lake Froylandsvatn)	Microcystins	Heptapeptides MW 1009	300-500
Oscillatoria agardhii var. (Waterbloom, Norway, Lake Kolbotnvatn)	Microcystins	Heptapeptides MW 1023	500-1000
Cytotoxins			
Scytonema pseudohofmanni Strain PC-1-3	Scytophycin A & B	Methylformamide A-MW 821; B-MW 819	650 (Scytophycin B)

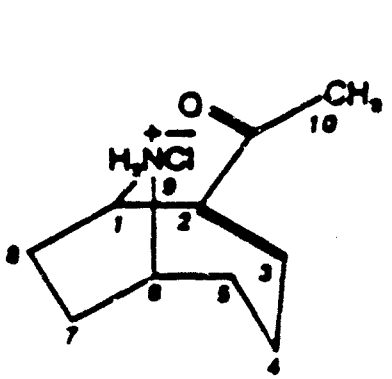
Scytonema hofmanni Strain UTEX-1581 (U.S., Texas)	Cyanobacterin	Chlorinated diaryllactone	not reported
Hapalosiphon fontinalis Strain V-3-1 (Marshall Islands)	Hapalindole A	Substituted indole alkaloid	not reported
Tolypothrix byssoidea Strain H-6-2 (U.S., Hawaii)	Tubercidin	Pyrrolopyrimidine	not reported
Oscillatoria acutissima Strain B-1 (U.S., Hawaii)	Acutiphycin	Macrolide	not reported

*See text for explanation of terminology.

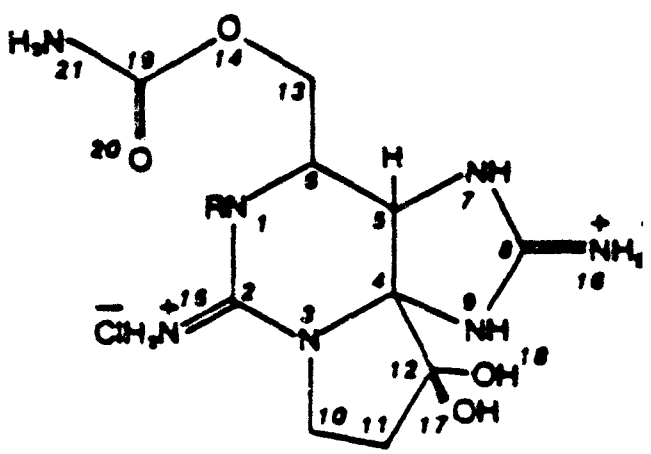
2. NEUROTOXINS

a. Anatoxins

Neurotoxins produced by filamentous Anabaena flos-aquae are called anatoxins (ANTX) (Carmichael and Gorham, 1978). Two anatoxins [ANTX-A and A(S)] are available for structure and function studies. ANTX-A from strain NRC-44-1 is the first toxin from a freshwater cyanobacteria to be chemically defined. It is the secondary amine, 2-acetyl-9-azabicyclo (4-2-1) non-2-ene (Huber, 1972; Devlin et al., 1977), molecular weight 166 daltons (Fig. 1a). It has been synthesized through a ring expansion of cocaine (Campbell et al., 1977, 1979), from iminium salts (Bates and Rapoport, 1979; Peterson et al., 1984, 1985), from 4-cycloheptenone or tetrabromotricyclooctane (Danheiser et al., 1985) by construction of the azabicyclo ring from 9-methyl-9-azabicyclo [3.3.1] nonan-1-ol (Wiseman and Lee, 1986), and by starting with 9-methyl-9-aza[4.2.1] nonan-2-one (Lindgren et al., 1987).



anatoxin - a hydrochloride



R=H; saxitoxin dihydrochloride
R=OH; neosaxitoxin dihydrochlorid

Fig. 1.a. (left) Anatoxin-a (ANTX-A) hydrochloride. Produced by the freshwater filamentous cyanobacterium Anabaena flos-aquae NRC-44-1.

(right) Aphantoxin-I (neosaxitoxin) and Aphantoxin-II (saxitoxin) produced by certain strains of the filamentous cyanobacterium Aphanizomenon flos-aquae.

ANTX-A is a potent, postsynaptic, depolarizing, neuromuscular blocking agent that affects both nicotinic and muscarinic acetylcholine (ACH) receptors at the ACH channel (Carmichael et al., 1979; Spivak et al., 1980, 1983; Aronstam and Witkop, 1981). Signs of poisoning in field reports for wild and domestic animals include staggering, muscle fasciculations, gasping, convulsions, and opisthotonos (birds). Death by respiratory arrest occurs within minutes to a few hours depending on species, dosage, and prior food consumption. The LD_{50} intraperitoneal (IP) mouse for purified toxin is about 200µg/kg body weight, with survival time of 4-7 min. This means that animals need to ingest only a few milliliters to a few liters of the toxic surface bloom to receive a lethal bolus (Carmichael and Gorham, 1977; Carmichael et al., 1977, Carmichael and Biggs, 1978).

Anatoxin-A(S) [ANTX-A(S)], produced by A. flos-aquae NRC-525-17, is different from ANTX-A. It produces opisthonos in chicks, as does ANTX-A, but also causes viscous salivation [which gives the terminology its (S) label] and lachrymation in mice, chromodacryorrhea in rats, urinary incontinence, and defecation prior to death by respiratory arrest. Also observed is a dose-dependent fasciculation of limbs for 1-2 min after death. ANTX-A(S) has been purified by column chromatography and high-performance liquid chromatography (HPLC) (Carmichael and Mahmood, 1984), but its structure is still being worked on. ANTX-A(S) is acid stable, unstable in basic conditions, has very low ultraviolet (uv) absorbance, gives a positive alkaloid test, and has a molecular weight estimated by gel exclusion chromatography and mass spectrometry of about 250 daltons.

The LD_{50} IP mouse for ANTX-A(S) is about 30 µg/kg, six times more toxic than ANTX-A. At the LD_{50} the survival time for mice is 10-30 min. Mahmood and Carmichael (1986a) conclude that the toxicological and pharmacological signs of poisoning indicate excessive, cholinergic stimulation. Recent work by Mahmood and Carmichael (1987) shows that ANTX-A(S) is an irreversible anticholinesterase.

Mahmood and co-workers (1988) have identified ANTX-A(S) as the probably cause of death for five dogs, eight pups and two calves that ingested quantities of A. flos-aquae in Richmond Lake, South Dakota, in late summer 1985. At present all neurotoxic A. flos-aquae strains studied in the laboratory have come from North America. There are, however, some recent reports of neurotoxic Anabaena in Australia (Runnegar et al., 1988a), Japan and Scandinavia (M. Watanabe, O.M. Skulberg, and K. Sivonen personal communication). It seems likely that once they are looked for, neurotoxic Anabaena will be found in all the same geographic areas as other toxic cyanobacteria.

b. Aphantoxins

Occurrence of neurotoxins (aphantoxins) in the freshwater filamentous cyanobacterium Aphanizomenon flos-aquae was first demonstrated by Sawyer and co-workers (1968). All aphantoxins (APHTXS) studied to date have come from waterblooms and laboratory strains of nonfasciculate (non-flake-forming) Aph. flos-aquae that occurred in lakes and ponds of New Hampshire from 1966 through 1980. Toxic cells and extracts of Aph. flos-aquae were shown to be toxic to mice, fish, and waterfleas (Daphnia catawba) by Jakim and Gentile (1968). Chromatographic and pharmacological evidence established that APHTXS consist mainly of two neurotoxic alkaloids that strongly resembled saxitoxin (STX) and neosaxitoxin (neoSTX), the two primary toxins of red tide paralytic shellfish poisoning (PSP) (Sasner et al., 1984). The bloom material and toxic strain used in studies before 1980 came from collections made between 1960 and 1970. The more recent work on APHTXS has used two strains (NH-1 and NH-5) isolated by Carmichael in 1980 from a small pond near Durham, New Hampshire (Carmichael, 1982; Ikawa et al., 1982). These APHTXS, as well as neoSTX and STX, are fast-acting neurotoxins that inhibit nerve conduction by blocking sodium channels without affecting permeability to potassium, and transmembrane resting potential, or membrane resistance (Adelman et al., 1982). Mahmood and Carmichael (1986b), using the NH-5 strain showed that batch-cultured cells have a mouse IP LD_{50} of about 5 mg/kg. Each gram of lyophilized cells yields about 1.3 mg aphantoxin I (neosaxitoxin) and 0.1 mg aphantoxin II (saxitoxin) (Fig. 1b). Also detected were three unstable neurotoxins that were not similar to any of the known paralytic shellfish poisons.

Shimizu and co-workers (1984) studied the biosynthesis of the STX analog neoSTX using Aph. flos-aquae NH-1. They were able to confirm its presence in strain NH-1 and to explain the biosynthetic pathway for this important group of secondary chemicals.

3. HEPATOTOXINS

Low-molecular-weight peptide toxins that affect the liver have been the predominant toxins involved in cases of animal poisonings due to cyanobacterial toxins (Schwimmer and Schwimmer, 1968; Carmichael, 1986; and Gorham and Carmichael, 1988). After almost 25 years of structure analysis on toxic peptides of the colonial bloom-forming cyanobacterium Microcystis aeruginosa, Botes and co-workers (1982a,b, 1986) and Santikarn and colleagues (1983) provided structure details on one of four toxins (designated toxin BE-4) produced by the South African M. aeruginosa strain WR70 (= UV-010). They concluded that it was monocyclic and contained three D-amino acids--alanine, erythro-β-methylaspartic acid, and glutamic acid, two L-amino acids--leucine and alanine--plus two unusual amino acids. These were N-methyldehydroalanine (Medha) and a nonpolar side chain of 20 carbon atoms that turned out to be a novel β-amino acid: 3-amino-

9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (ADDA). Based on fast atom bombardment mass spectrometry (FABMS) and nuclear magnetic resonance (NMR) studies, BE-4 toxin is now known to be a cyclic heptapeptide having a molecular weight of 909 daltons. Botes and co-workers (1985) also showed that the other three toxins of strain WR-70 all had the same D-amino acids and the two novel amino acids (Medha and ADDA). They differed in that the L-amino acids were leucine-arginine; tyrosine-arginine and tyrosine-alanine instead of leucine-alanine as in toxin BE-4. They were also able to show that the hepatotoxin isolated by Elleman and colleagues (1978) from water bloom material collected in Malpas Dam, New South Wales, Australia, contained the five characteristic amino acids plus the L-amino acid variants tyrosine-methionine.

Instead of calling the BE-4 toxin microcystin, as previous Microcystis toxins were called (Konst et al., 1965; Murthy and Capindale, 1970; Rabin and Derbre, 1975) and using alphabetical or numerical suffixes to indicate chromatographic elution order or structural differences, Botes (1986) proposed the generically derived designation cyanoginosin (CYGSN). This name, which indicates the cyanobacterial species (i.e. aeruginosa) origin, is followed by a two-letter suffix that indicates the identity and sequence of the two L-amino acids relative to the N-Me-dehydroalanyl-D-alanine bond. Thus toxin BE-4 was renamed cyanoginosin-LA since leucine and alanine are the L-amino acids.

Microcystin (MCYST) is the term given to the fast death factor (FDF) produced by M. aeruginosa strain NRC-1 and its daughter strain NRC-1 (SS-17) (Bishop et al., 1959; Konst et al., 1965). A definitive structure for the toxin of strain NRC-1 (SS-17) is not yet available but is known to be a peptide (MW 994) containing the variant L-amino acids leucine and arginine (Carmichael, unpublished). Krishnamurthy and co-workers (1986a,b) have shown that the toxin isolated from a waterbloom of M. aeruginosa collected in Lake Akersvatn, Norway (Berg et al., 1987), has a structure similar to that of MYCST from NRC-1 (SS-17) and CYGSN-LR. This toxin has also been found to be the main toxin produced by the Scottish strain of M. aeruginosa PCC-7820 and a Canadian A. flos-aquae strain S-23-g-1 (Krishnamurthy et al., 196 a,b). The identification of a peptide toxin from A. flos-aquae S-23-g-1 provides the first evidence that these hepatotoxins are produced by filamentous as well as coccoid cyanobacteria. A. flos-aquae S-23-g-1 and toxic M. aeruginosa from a waterbloom in Wisconsin also produced a second cyclic heptapeptide hepatotoxin, which has been found to have six of the same amino acids, that is, leucine-arginine, but has aspartic acid instead of β-methylaspartic acid (Krishnamurthy et al., 1986a).

The filamentous genus Oscillatoria has also been shown to produce a hepatotoxin (Ostensvik et al., 1981; Eriksson et al., 1987a). From water blooms of O. agardhii var and O. agardhii var. isothrix, two similar cyclic heptapeptides have been isolated. Both toxins have the variant L-amino acids arginine-arginine and

aspartic acid instead of β-methylaspartic acid. The toxin from O. agardhii var. isothrix also has dehydroalanine instead of methyldehydroalanine (Krishnamurthy et al., 1986b). More recently M. viridis (Kusumi et al., 1987) and M. aeruginosa (Painuly et al., 1988; Harada et al., 1988) have been shown to produce the cyclic heptapeptide with an arginine-arginine "L" amino acid variant.

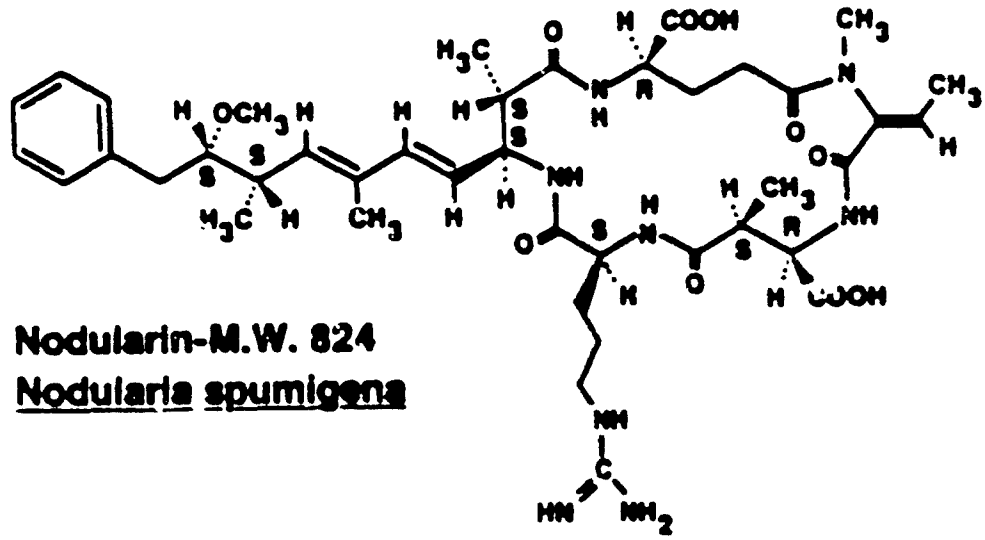
Nodularia spumigena has also been shown to produce a peptide with hepatotoxic activity. The more recent reports come from Australia (Main et al., 1977), the German Democratic Republic (Kalbe and Tiess, 1964), Denmark (Lindstrom, 1976), Sweden (Edler et al., 1985) and Finland (Eriksson et al., 1988; Persson et al., 1984). Recently structure information on Nodularia toxin has been presented by Rinehart (Royal Soc. Chem., Ann. Chem. Congress, Swansea, U.K., April 13-16, 1987, Paper A-12) for waterbloom material collected in Lake Forsythe, New Zealand in 1984; by Carmichael and co-workers (1988) for a clonal isolate from Lake Ellesmere, New Zealand; by Eriksson and co-workers (1988) from waterbloom material collected in the Baltic Sea in 1986 and Runnegar and colleagues (1988b) for a field isolate from the Peel Inlet, Perth, Australia. Structure work by these groups all indicate that the peptide is smaller than the heptapeptides toxins. Rinehart and co-workers (1988) showed that the toxin is a pentapeptide with a similar structure to the heptapeptides and containing β-methylaspartic acid, glutamic acid, arginine, N-methyl-dehydrobutyrine and ADDA (M.W. 824) (Fig. 2).

1. Mode of Action for Microcystins

The liver has always been reported as the organ that showed the greatest degree of histopathological change when animals are poisoned by these cyclic peptides. The molecular basis of action for these cyclic peptides is not yet understood but the cause of death from toxin and toxic cells administered to laboratory mice and rats is at least partially known and is concluded to be hypovolemic shock caused by interstitial hemorrhage into the liver (Theiss et al., 1988). This work with small animal models is currently being extended to larger animals in order to study the uptake, distribution, and metabolism of the toxins (Beasley et al., unpublished data). There is evidence to show from studies using ^{125}I-labeled CYGSN-YM (MCYST-YM) that the liver is the organ for both accumulation and excretion (Falconer et al., 1986; Runnegar et al., 1986a). Brooks and Codd (1987), using C^{14} labeled MCYST-LR, showed that seventy percent of the labeled toxin was localized in the mouse liver after 1 min following intraperitoneal injection of the toxin.

Studies at both the light and electron microscopic (EM) level of time-course histopathological changes in mouse liver show rapid and extensive centrilobular necrosis of the liver with loss of characteristic architecture of the hepatic cords.

Fig. 2 Structure of nodularin (NODLN) produced by Nodularia spumigena waterbloom from Lake Forsythe, New Zealand and clonal isolate L575 from Lake Ellesmere, New Zealand (Rinehart et al., 1988).



Sinusoid endothelial cells and then hepatocytes show extensive fragmentation and vesiculation of cell membranes (Runnegar and Falconer, 1981; Foxall and Sasner, 1981). Using microcystin-LR from M. aeruginosa strain PCC-7820, Dabholkar and Carmichael (1987) found that at both lethal and sublethal toxin levels hepatocytes show progressive intracellular changes beginning at about 10 min postinjection. The most common response to lethal and sublethal injections is vesiculation of rough endoplasmic reticulum (RER), swollen mitochondria, and degranulation (partial or total loss of ribosomes from vesicles). The vesicles appear to form from dilated parts of RER by fragmentation or separation. Affected hepatocytes remain intact and do not lyse. Use of the isolated perfused rat liver to study the pathology of these toxins shows similar results to the in vivo work. Berg and co-workers (1988) used three structurally different cyclic heptapeptide hepatotoxins (MCYST-LR; desmethyl MCYST-RR and didesmethyl MCYST-RR). All three toxins had a similar effect on the perfused liver system although both "RR" toxins required higher concentrations (5-7x) to produce their effect. This was consistent with the lower toxicity of the "RR" toxins, which was about 500 and 1000 µg/kg i.p. mouse compared to 50 µg/kg for MCYST-LR.

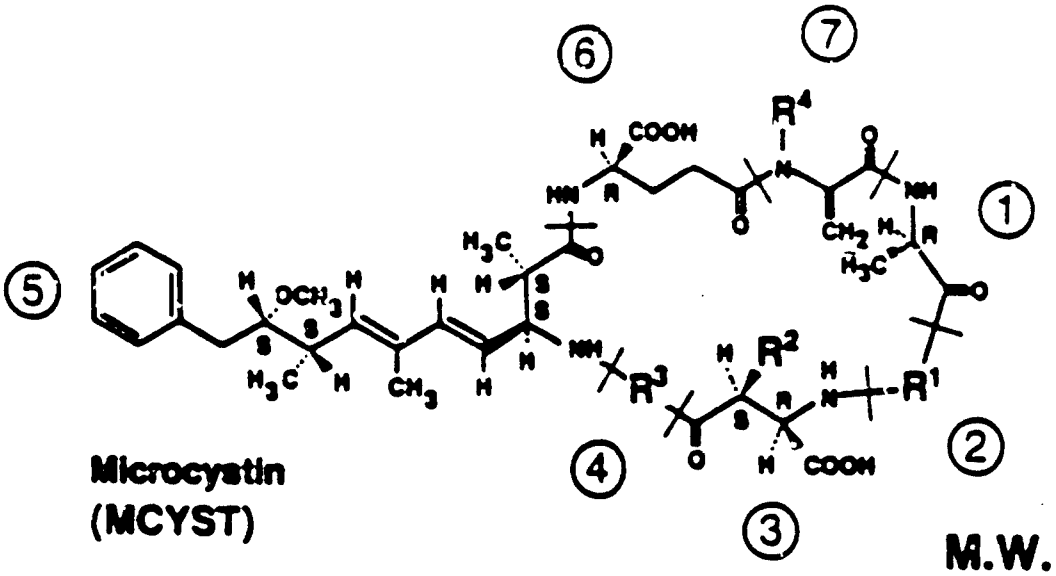
In vitro studies on isolated cells including hepatocytes, erythrocytes, fibroblasts and alveolar cells continue to demonstrate the specificity of action that these toxins have for liver cells (Eriksson et al., 1987a; Runnegar et al., 1987 and Falconer and Runnegar, 1987). This has led Aune and Berg (1987) to use isolated rat hepatocytes as a screen for detecting hepatotoxic waterblooms of cyanobacteria.

The cellular/molecular mechanism of action for these cyclic peptide toxins is now an area of active research in several laboratories. these peptides cause striking ultrastructural changes in isolated hepatocytes (Runnegar and Falconer, 1986b) including a decrease in the polymerization of actin. This effect of the cells cytoskeletal system continues to be investigated and recent work supports the idea that these toxins interact with the cells cytoskeletal system (Eriksson et al., 1987b; Falconer and Runnegar, 1987). The apparent specificity of these toxins for liver cells is not clear although it has been suggested that the bile uptake system may be at least partly responsible for penetration of the toxin into the cell (Berg, et al., 1988).

2. Naming the Cyclic Peptide Hepatotoxins

The hepatotoxins have been called Fast-Death Factor (Bishop et al., 1959), Microcystin (Konst et al., 1965), Cyanoginosin (Botes et al., 1986), Cyanoviridin (Kusumi et al., 1987) and Cyanogenosin (apparently a misspelling of cyanoginosin) (Painuly et al., 1988). Continued use of this multiple naming system will create confusion and misunderstanding as more is published on these cyclic peptides. A number of investigators doing research on these toxins have therefore proposed a system of nomenclature based on the original term microcystin (MCYST) (Carmichael et

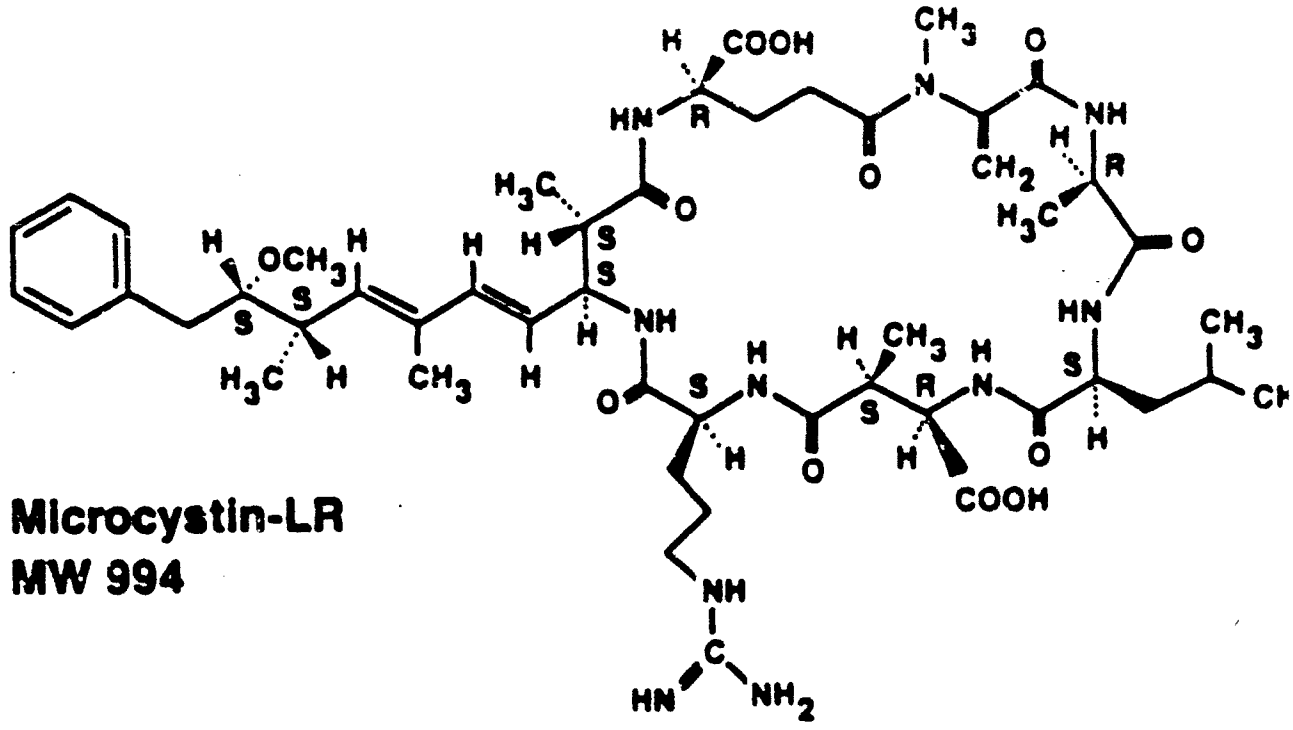
al., 1988, Appendix II). Using this system the structures of known microcystins are given in Fig. 3.



	MCYST-LA: R^1 = Leu; R^2 = CH_3; R^3 = Ala; R^4 = CH_3	909
	MCYST-YA: R^1 = Tyr; R^2 = CH_3; R^3 = Ala; R^4 = CH_3	959
	MCYST-LR: R^1 = Leu; R^2 = CH_3; R^3 = Arg; R^4 = CH_3	994
desmethyl 3-	MCYST-LR: R^1 = Leu; R^2 = H; R^3 = Arg; R^4 = CH_3	980
	MCYST-YM: R^1 = Tyr; R^2 = CH_3; R^3 = Met; R^4 = CH_3	1035
	MCYST-RR: R^1 = Arg; R^2 = CH_3; R^3 = Arg; R^4 = CH_3	1037
desmethyl 3-	MCYST-RR: R^1 = Arg; R^2 = H; R^3 = Arg; R^4 = CH_3	1023
desmethyl 3,7-	MCYST-RR: R^1 = Arg; R^2 = H; R^3 = Arg; R^4 = H	1009
	MCYST-YR: R^1 = Tyr; R^2 = CH_3; R^3 = Arg; R^4 = CH_3	1044

Fig. 3 Structure of known microcystins (refer also to Table 2).

a. Structure of six microcystins varying only in L-amino acids and three microcystins with desmethyl portions of amino acids 3 and 7



b. Structure of Microcystin-LR, the most commonly found toxin in this group.

B. WORKSCOPE (Experimental Programs)

1. Culture, Harvest, Cell Yields, Field Sampling, and Preservation of Toxic Blue-Green Algae (Cyanobacteria)

Anabaena flos-aquae strains NRC-525-17-b-1-e and 44-1-S, and Microcystis aeruginosa strains PCC-7820 and M-228 are being grown in bulk quantities to provide material for the extraction of Anatoxin-a(s), Anatoxin-a, Microcystin-LR and YR, the toxins produced respectively by these four cyanobacteria. Nodularia spumigena strain L-575 and Aphanizomenon flos-aquae strain NH-5-a are being grown in moderate bulk quantities to provide material for toxin analysis. Twenty-five 20-liter flasks with stirring paddles, and one 90-liter plexiglass cylinder are being used to grow M. aeruginosa 7820 in a semi-continuous batch culture system. Two 20-liter flasks with stirring paddles and one 180-liter fiberglass cylinder are being used to grown M. aeruginosa M-228 in a semi-continuous batch culture system. N. spumigena L-575 and Aph. flos-aquae NH-5-a are being batch cultured in five and nine 12-liter bottles, respectively. A. flos-aquae 44-1-S is being semi-continuously cultured in one 20-liter flask. Two 20-liter flasks are dedicated to growing another neurotoxic strain of A. flos-aquae (IG-20) for toxin analysis. One 90-liter computer-monitored fermenter was installed in January of 1988 for semi-continuous culturing of 525-17-b-1-e.

A. flos-aquae strain 525-17-b-1-e is being batch cultured in two 200-liter tubs, using room temperature conditions. These fiberglass tubs were prepared for culture by sealing their inside surfaces with polyurethane. A 5/8" PVC pipe studded with 5 aquarium aerators is wedged lengthwise into the bottom of each tub. Filtered room air is used to aerate the cultures (Whatman 12-20 grade filter tubes). Each tub is covered by a sheet of plexiglass elevated slightly above the top of the tub by rubber stoppers at each corner. Banks of four 4-foot Duro-Test Vita-Lites (40 watts) are suspended above each tub. The incident light passing through the plexiglass and reaching the surface of the culture is 80-100 $\mu E/m^2/s$. The medium used is BG-11. The medium is prepared by first filling the tubs with deionized water that has been filter-sterilized through a 0.22 μ Millipack 200 filter unit. Nutrient salts are dissolved separately in 1- or 2-liter flasks, autoclaved, and then added to the water filled tubs. Aeration is used to mix the contents, inoculum (12 liters) is added, allowed to mix, and then the air and light are removed overnight. More inoculum may be added later, depending on the growth of the culture. The total contents of the tubs are harvested every three weeks. The 200 liter contents of each tub are reduced during harvesting to about 3 liters with a Pellicon Millipore cell concentrator system. Concentration is done in about four hours, with cell recovery over 95%. If the cells are healthy and the culture is not lysing, the toxin is retained within the cells. The concentrated cells are freeze-dried and stored in a -18°C freezer until they are extracted.

A. flos-aquae strain 525-17-b-1-e is also semi-continuously cultured in five 180-liter cylinders at 22-25°C. These cylinders are of two types: four have 5/8" PVC pipes with air holes drilled into the bottom 6 inches extending the length of the cylinders; one has custom made aerators made of plexiglass and amber latex tubing at the base of the cylinders. Banks of two 4-foot Duro-Test Vita-Lites (40 watts) are suspended beside the cylinders. The incident light passing through the fiberglass and reaching the surface of the cultures is 80-100 $\mu E/m^2/s$. Filtered room air is used to aerate the cultures (Whatman 12-20 grade filter tubes and Millex-FG 0.2 µm filter units). The medium used is BG-11. Medium and nutrient salts are added to the cylinders in the same way that they are added to the fiberglass tubs. Inoculum (24 liters) is added and allowed to mix. Aluminum foil around the cylinders is used to regulate the incident light, especially during the first 1 or 2 weeks of growth. More inoculum may be added 2 or 3 days later, depending on the growth of the culture. These cylinders are harvested once per week by removing 24 liters. Sampling of the cultures is done via a stopcock at the base of the cylinder, and replacement of the volume is done with sterile BG-11 medium poured into the top of the cylinder. The 24 liter sample is reduced to approximately 3 liters with the Pellicon Cell Concentrator. The concentrated cells are freeze-dried and stored.

Since Microcystis aeruginosa strain 7820 experiences a 3-4 day lag period when cultured, it is semi-continuously cultured in 25 20-liter Bellco Microcarrier Magnetic stirrers. These flask cultures are kept at 22-25°C. The flasks are illuminated with Vita-Lite fluorescent bulbs. Cultures in the flasks are aerated with filtered room air passed through glass aerators. The aerators are inserted into Consolidated Plastics bulkhead unions on the left side of the spinner flasks. The stem of the spinner flask stirring blade is held by a bulkhead union in the center cap of the flasks. The right hand cap holds a glass elbow vent tube. The culture is sampled and medium (BG-11) aseptically replaced through this tube. Various harvest volumes and lengths of time between harvests were compared to find the most productive combination without depressing growth of the culture. Presently, 8-9 liters are taken once per week from each flask. Initial set-up of the flasks involves autoclaving about 12 liters of BG-11 medium in each flask and inoculating it with 4 liters of log phase culture. Sampling of the cultures and replacement of volume with sterile media is done by syphoning out the algae and draining in the sterile media from an elevated 9 liter jar through the glass elbow tube. Cells harvested from all jars on a given date are combined, concentrated, and freeze-dried. Freeze-dried material is stored at -18°C until it is extracted.

Microcystis aeruginosa 7820 is also batch cultured in one 90-liter plexiglass cylinder kept at 22-25°C. A 1/8" PVC pipe with air holes drilled into the bottom 6" extends the length of the cylinder. Filtered room air is used to aerate the culture. Banks of 40 watt Vita-Lites provide incident light. The medium used is BG-11. Sixty-eight liters of sterile BG-11 is added to

the cylinder by pouring it into the top. This is allowed to mix by aeration. Inoculum (12 liters) is added and allowed to mix. Additional inoculum may be added later depending on the growth of the culture. The total contents of the cylinder are harvested every 4-5 weeks. The approximately 90 liters are reduced to about 3 liters, freeze-dried, and stored at -18°C. Microcystis aeruginosa strain M-228 is being grown in one 180-liter cylinder which is managed as the other 180-liter cylinders, with BG-11 used as the medium. It is also grown in two 20-liter Bellco spinner flasks. Again, these flasks are managed as the other 20-liter spinner flasks.

Presently, A. flos-aquae strains 44-1-s and 1G-20 are being grown in one and two 20-liter Bellco Spinner flasks, respectively. Both are managed as the other 20-liter spinner flasks, with ASM-1 used as the medium.

N. spumigena L-575 and Aph. flos-aquae NH-5-a are batch cultured in five and nine 12-liter bottles, respectively. The cultures are kept at 22-25°C. They are illuminated with Vita-Lite fluorescent bulbs and aerated with filtered air passed through glass aerators. Presently, the total volumes of five L-575 12-liter bottles are harvested every 2-3 weeks. These volumes are reduced to 3 liters, freeze-dried, and stored at -18°C. Table 3 and Table 4 summarize current culture volumes, LD_{50}'s, and yields of cells.

Table 3. Summary of Volumes Currently Being Used for Cultures of Cyanobacteria

Code	Name of Cyanobacteria	Source of Culture	Current Culture Volume (L)	Average Yield of cells (g/L/week)	Toxin	LD_{50} (mg/kg)
7820	M. aeruginosa	P.C.C.	470	0.2532	Microcystin LR	<100
M-228	M. aeruginosa	Japan	210	0.1758	Microcystin LR + YR	<100
525-17-b-1-e	A. flos-aquae	N.R.C.	1350	0.1948	Anatoxin-a(s)	<100
IG-20	A. flos-aquae	Illinois	30	0.1238	Anatoxin-a(s)	<100
44-1-s	A. flos-aquae	N.R.C.	39	0.1535	Anatoxin-a	>250
L-575	N. spumigena	New Zealand	60	1.4606	Hepatotoxin	<50
NH-5-a	Aph. flos-aquae	New Hampshire	110	0.2941	Neurotoxin (Aphantoxin)	<5
Gleotrichia	Gleotrichia	Montana	1	0.4710	----	not yet tested

P.C.C. - Pasteur Culture Collection, Paris, France
Illinois - field isolate, Griggsville, Illinois
Japan - field isolate
N.R.C. - National Research Council, Ottawa, Canada
New Zealand - field isolate
New Hampshire - field isolate
Montana - field isolate

Table 4 Quarterly Summary of Dry Weight Cell Yields
(October 1987 - October 1988)
(grams/Liter)

Culture	1st Quarter Oct 87 - Dec 87	2nd Quarter Jan 88 - Mar 88	3rd Quarter Apr 88 - Jun 88	4th Quarter Jul 88 - Sep 88	TOTALS
/820	111.9 g/879 L	163.3 g/1230 L	306.4 g/2078 L	284 5 g/2303 L	865 9 g/6490 L
M-228	2.9 g/10 L	18 4 g/156 L	89.4 g/570 L	37.4 g/252 L	148 0 g/988 L
525-17-b-1-E	119.2 g/1880 L	89 9 g/1219 L	117 3 g/1862 L	186.9 g/2936 L	513 2 g/7897 L
IG-20	17.2 g/162 L	10 6 g/102 L	10 6 g/76 L	13.0 g/108 L	51 2 g/448 L
44-1-a	17 4 g/251 L	18.9 g/117 L	15.2 g/98 L	....	51 5 g/466 L
L-575	37.2 g/52 L	120 8 g/92 L	233.9 g/464 L	278.3 g/664 L	670 1 g/1272 L
NH-5-a	....	....	6.3 g/30 L	18.3 g/60 L	24 6 g/90 L
Gleotrichia	....	....	....	3.8 g/8 L	3 8 g/8 L

2. Timeline for culturing and harvesting toxic cyanobacteria.

a. Timeline involved in growing batch cultures of *A. flos-aquae* NRC 525-17 in 200-liter tubs.

About 15 days	1)	Growing 25 ml Delong flasks
About 15 days	2)	Growing 1-L Delong flasks
About 30 days	3)	Growing 4-L Delong flasks
About 45 days	4)	Growing 12-L bottles
About 2 hrs/3 wks	5)	Cleaning, sterilizing two 200-L tubs
About 3 hrs/3 wks	6)	Filter-sterilizing water for tub cultures
About 2 hrs/3 wks	7)	Preparing media for the tubs
Ten to twenty min	8)	Inoculating the tubs
About 21 days	9)	Allowing the cultures to grow
About 4 hours	10)	Harvesting the tub cultures
About 44-48 hours	11)	Freeze-drying the harvested cells
About 1 hr/3 wks	12)	Bottling, storing, & logging the dried cells

b. Timeline involved in growing semi-batch cultures of *A. flos-aquae* NRC-525-17 in 180-liter cylinders

About 15 days	1) Growing 25 ml Delong flasks
About 15 days	2) Growing 1-L Delong flasks
About 30 days	3) Growing 4-L Delong flasks
About 45 days	4) Growing 12-L bottles
About 2 hrs/2 mos	5) Cleaning, sterilizing each 180-L cylinder
About 2 hrs/2 mos	6) Filter-sterilizing water for the cylinders
About 2 hrs/2 mos	7) Preparing media for the cylinders
Ten to twenty min	8) Inoculating the cylinders
About 30 days	9) Allowing the cultures to grow
About 3 hours	10) Harvesting 24 liters from the cylinders
About 44-48 hours	11) Freeze-drying the harvested cells
About 1 hr/3 wks	12) Bottling, storing, & logging the dried cells

c. Timeline for growing *M. aeruginosa* PCC 7820 in semi-batch (20-liter) and batch (90-liter) cultures

About 45 days	1)	Growing inoculum for 20 L & 90 L cultures
About 2-3 hrs/vessel	2)	Sterilizing & settling up the culture vessels
About 6 hrs/wk	3)	Preparing media for the 20 L flasks
About 7 days	4)	Allowing 20 L cultures to grow
About 4-5 wks	5)	Allowing 90 L cylinder to grow
About 3 hrs/wk	6)	Collecting 8 L from each flask
About 3 hrs/4-5 wks	7)	Harvesting @ 90 L from 90 L cylinder
About 2 hrs/wk	8)	Replacing media in flasks
Few minutes	9)	Replacing media in the cylinder
About 4 hrs/wk	10)	Concentrating the cells from the flasks
About 2 hrs/4-5 wks	11)	Concentrating the cells form the cylinders
About 44-48 hrs	12)	Freeze-drying the harvested cells
About 1 hr/wk	13)	Bottling, storing, and logging the dried cells

d. Timeline for growing semi-batch cultures of *M. aeruginosa* M-228 in 20-liter flasks and in 180-liter cylinders

-- very similar to that of 525-17-b-1-e in 180-liter cylinders and of 7820 in 20-liter flasks

e. Timeline for growing batch cultures of L-575 and NH-5-a

About 15 days	1)	Growing 25 mL Delong flasks
About 15 days	2)	Growing 1-L Delong flasks
About 30 days	3)	Growing 4-L bottles
About 45 days	4)	Growing 12-L bottles
About 2 hours	5)	Harvesting total volume of bottles
About 44-48 hours	6)	Freeze-drying the harvested cells
About 1 hr/1 wk	7)	Bottling, storing, & logging the dried L-575 cells
About 1 hr/2-3 wks	8)	Bottling, storing, & logging the dried NH-5-a cells

f. Timeline for growing semi-batch cultures of *A. flos-aquae* strains 44-i-s and IG-20 in 20-liter flasks

-- very similar to that of 7820 in 20-liter flasks with harvest occurring every two weeks.

Table 5. Culture Media for Growth of Toxic Cyanobacteria

Nutrient	ASM-1 (mg/L)	BG-11 (mg/L)	BG-11 (L-575)	BG-11 (Gleotrichia) (mg/L)	z-8 (mg/L)	z-8 with salt (mg/L)	z-8 without nitrogen (mg/L)
$NaNO_3$	170.00	1500.00	750.00	1500.00	467.00	467.00	---
K_2HPO_4	17.40	40.00	40.00	40.00	31.00	31.00	31.00
Na_2HPO_4	14.20	---	---	---	---	---	---
$MgCl_2$	19.02	---	---	---	---	---	---
$MgSO_4 \cdot 7H_2O$	49.32	75.00	75.00	75.00	25.00	3775.00	25.00
$CaCl_2 \cdot 2H_2O$	29.40	36.00	36.00	36.00	---	---	37.00
Citric Acid	---	6.00	6.00	6.00	---	---	---
Na_2CO_3	---	20.00	20.00	20.00	21.00	21.00	21.00
Na_2EDTA	6.64	1.00	1.00	1.00	---	---	---
Ferric Ammonium Citrate	---	6.00	6.00	6.00	---	---	---
NaCl	---	---	7000.00	---	---	8750.00	---
$Ca(NO_3)_2 \cdot 4H_2O$	---	---	---	---	59.00	59.00	---
Fe - EDTA	---	---	---	---	0.344	0.344	0.344

ASM-1 minor elements: (mg/kg in culture medium) $FeCl_3$ - 0.65, H_3BO_3 - 2.47, $MnCl_2 \cdot 4H_2O$ - 0.87, $ZnCl_2$ - 0.44; $CoCl_2 \cdot 6H_2O$ - 0.01, $CuCl_2 \cdot 2H_2O$ - 0.0001 (In our laboratory, Tris is added at the level of 26.90 mg/12 - this provides better buffering of the medium and it increases the length of storage for unused media). ASM-1 is adjusted to pH 8.5 with 0.5 NaOH before autoclaving.

BG-11 minor elements: (g/L) H_3BO_3 - 2.86, $MnCl_2 \cdot 4H_2O$ - 1.81, $ZnSo_4$ - 0.222, $Na_2MoO_4 \cdot 2H_2O$ - 0.39, $CuSo_4 \cdot 5H_2O$ - 0.079, $Co(NO_3)_2 \cdot 6H_2O$ - 0.049. Add 1 ml/L into the culture medium. After autoclaving and cooling, pH of the medium is about 7.1.

z-8 minor elements: (g/L) $Na_2WO_4 \cdot 2H_2O$ - 0.33, $(NH_4)_6 Mo_7O_{24} \cdot 2H_2O$ - 0.88, KBr - 1.20, KI - 0.83, $ZnSO_4 \cdot 7H_2O$ - 2.87, $Cd(NO_3)_2 \cdot 4H_2O$ - 1.55, $Co(NO_3)_2 \cdot 6H_2O$ - 1.46, $CuSO_4 \cdot 5H_2O$ - 1.25, $NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$ - 1.98, $Cr(NO_3)_3 \cdot 9H_2O$ - 0.41, V_2O_5 - 0.089, $Alz(SO_4)_3K_2SO_4 \cdot 24H_2O$ - 4.74, H_3BO_3 - 3.10, $MnSO_4 \cdot 4H_2O$ - 2.23

3. Recloning of A. flos-aquae NRc-44-1 -- Producer of Anatoxin A.

Single filament isolates from A. flos-aquae strain 44-1-s were done when it was found that the LD_{50} had risen greater than 250 and in some case was non-toxic. Isolates (usually varying in ability to produce toxin) were made in two ways:

1) Isolates were made by pipetting a few filaments from the culture onto a clean microscope slide. A drop of sterile media was added to the colonies; gentle blowing on the drop through a Pasteur pipet dispersed the colonies. A desired filament was located in the drop using an inverted compound microscope. The filament was then drawn into a Pasteur pipet by capillary action (the pipet tip was tapered on a flame to allow more accuracy in selecting a single filament). The single filament was transferred to a second drop of sterile media, gentle blowing was used to separate it from any other filaments or debris, and the filament was again transferred to another drop of sterile media. This was done two-four times. The selected filament was finally transferred to a culture tube containing 1-2 mL of sterile media. Each isolate was coded with the original culture name (i.e. 44-1-s) and a number designating its position in the total number of isolates made. Surviving isolates are currently being cultured and tested for toxicity.

2) Isolates were made by pipetting one drop of culture onto sterile agar in a petri-dish (ASM-1), which was then spread out across the surface of the agar. When the cyanobacterial cultures appeared on the agar, each individual colony was transferred to a culture tube containing 1-2 mL of sterile media. Each isolate was coded as in procedure #1. Surviving isolates are currently being cultured and tested for toxicity.

4. Isolation and Purification of Peptide Hepatotoxins

During the time period of this report 593 mg of microcystin-LR (cyanoginosin-LR) and 38 mg of Nodularia toxin (Nodularin) were supplied to USAMRIID (Table 6). the microcystin-LR samples included 518.3 mg isolated from bloom samples of M. aeruginosa strain PCC 7820. Nodularin toxin was isolated from laboratory cultures of N. spumigena strain L575.

Consistent toxicities are now being observed with cultures of M. aeruginosa strain PCC 7820. It has been noted in last year's report that there had been a partial loss in the toxicity per gram of cultured cells. The isolation of pure hepatotoxin from cultures of M. aeruginosa 7820 has been hampered by the presence of a contaminating pigment in the isolated toxin fraction. In previous isolations of this toxin pigment contamination was removed during HPLC chromatography. However, in current isolation procedures the contaminating pigment co-elutes with the toxin during TPLC chromatography. Neither a linear gradient of CH_3CN in 10 mM NH_4CH_3COO (0 to 50% over a 60 min period in a Waters Delta Pup

reversed phase C18 column) or an isocratic run of the % CH_3CN in water succeeded in removing the pigment from the toxic fraction. Future attempts to separate the pigment will include the preferential elution of the toxin, or the pigment, from Bond Elute silica cartridges with increasing concentrations of CH_3OH in CH_3Cl. Re-chromatography of the toxic fraction by gel filtration on Sephadex G-25 will also be checked as a method to remove the pigment from the toxin.

In last year's report it was noted that the purified toxin from M. aeruginosa strain 7820 consistently showed an asymmetrical peak on reversed phase HPLC. In addition amino acid analysis of the toxin showed two peaks representing both aspartic acid and β-methyl aspartic acid. The separation of the side peak was improved by both increased salt concentration and a decrease in pH of the aqueous solvent system. When a mobile phase of CH_3OH--50 mM phosphate buffer (pH 3.3, 6:4), described by Harada et al. (1988), was employed two distinct peaks were observed (Fig. 4). It should now be possible to determine if the side peak represents the demethylated toxin. The purification scheme for the isolation of the hepatotoxin from N. spumigena is presented in Fig. 5. Preparative scale isolation and shipment of this toxin to USAMRIID will be continued.

Table 6. Cyclic Heptapeptide Microcystin-LR and Pentapeptide Nodularia Supplied to USAMRIID (1C/87 - 10/88)

Date	Amount (mg)	Source
12/2/87	159.9	Monroe and Akersvatn water bloom lyophilized cells
3/15/88	75.2	Strain PCC 7820
5/26/88	250.0	Monroe water bloom
8/1/88	18.1	*N. spumigena* L575
9/2/88	19.6	*N. spumigena* L575
10/24/88	108.4	Monroe water bloom

Figure 4. HPLC profile of MCYST-LR from M. aeruginosa PCC-7820 showing side peak (left of large peak). This side peak is thought to be a desmethyl--MCYST-LR or a conformational isomer (perhaps in the ADDA portion of the molecule).

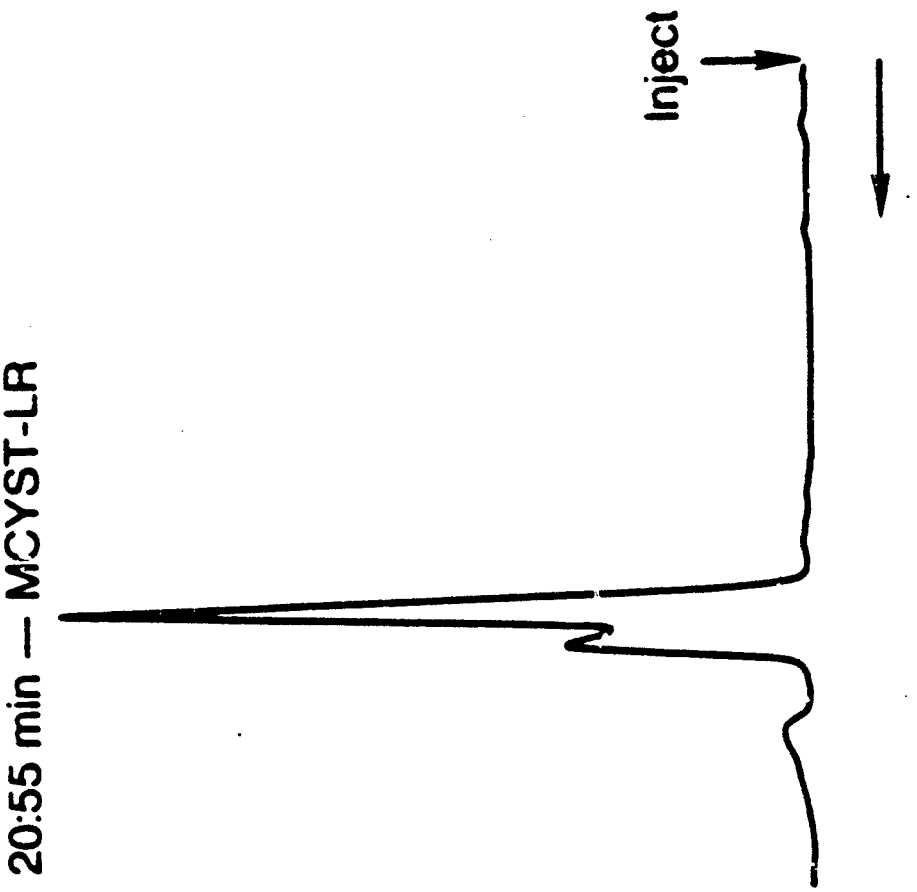
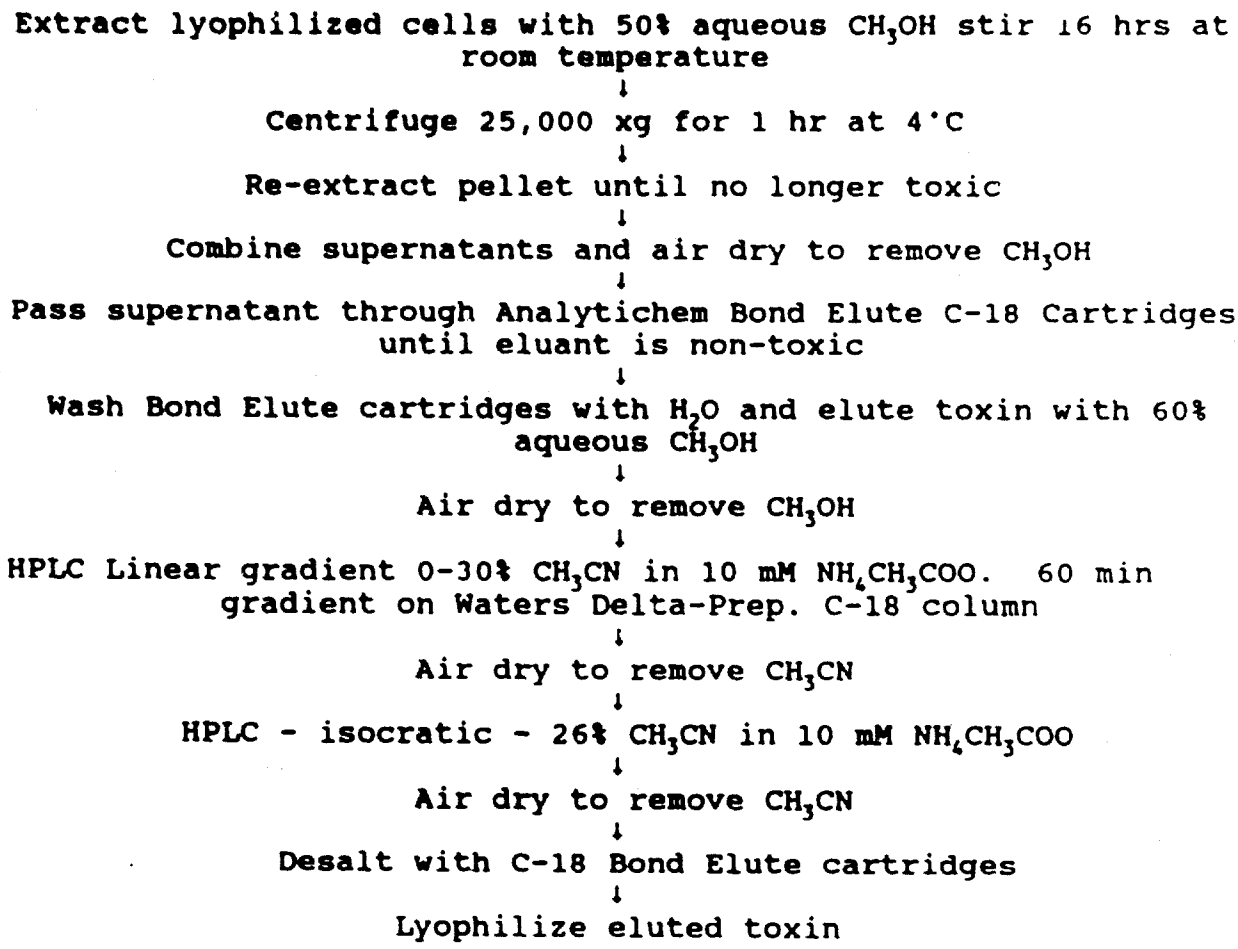


Figure 5. Purification Scheme for *N. spumigena* Toxin [Nodularin]



Cooperative studies with the Department of Microbiology, University of Helsinki, Finland have been undertaken. The focus of these studies is the purification and identification of the toxins present in toxic blue-green algae blooms from the Baltic Sea. The predominant species associated with these blooms has been identified as a Nodularia species (Edler et al. 1985). Amino acid analysis of the toxin isolated from these blooms indicated the presence of three amino acids; glutamate, β-methyl aspartate and arginine. These results are consistent with the previous identification of a toxic pentapeptide found in N. spumigena (Carmichael et. al. 1988 and Eriksson et al. 1988). In addition to the studies on the Nodularia toxin, work on the purification and identification of the toxins found in an isolate of a filamentous toxic Nostoc from Finnish freshwaters also initiated. Preliminary data indicates the possible presence of six different toxins in this blue-green algae culture.

Amino acid analysis and FAB-MS indicate that these hepatotoxins may be microcystin-RR, desmethyl-RR, di-desmethyl RR, LR, desmethyl LR, and LP. Additional analysis of these isolated toxins are in progress. The isolation and purification of the toxic peptides from an isolate of M. aeruginosa strain M-228 has also been initiated during this report period. This strain produces both the LR and YR cyclic peptides.

The LD_{50} of this strain in culture ranges from 50 to 100 mg/kg (i.p.) in male mice. A flow chart for the extraction and purification of LR and YR from M-228 is presented in Fig. 6. An HPLC profile of the purified toxins is shown in Fig. 7.

Figure 6. Purification Scheme for *M. aeruginosa* strain M228 Toxins

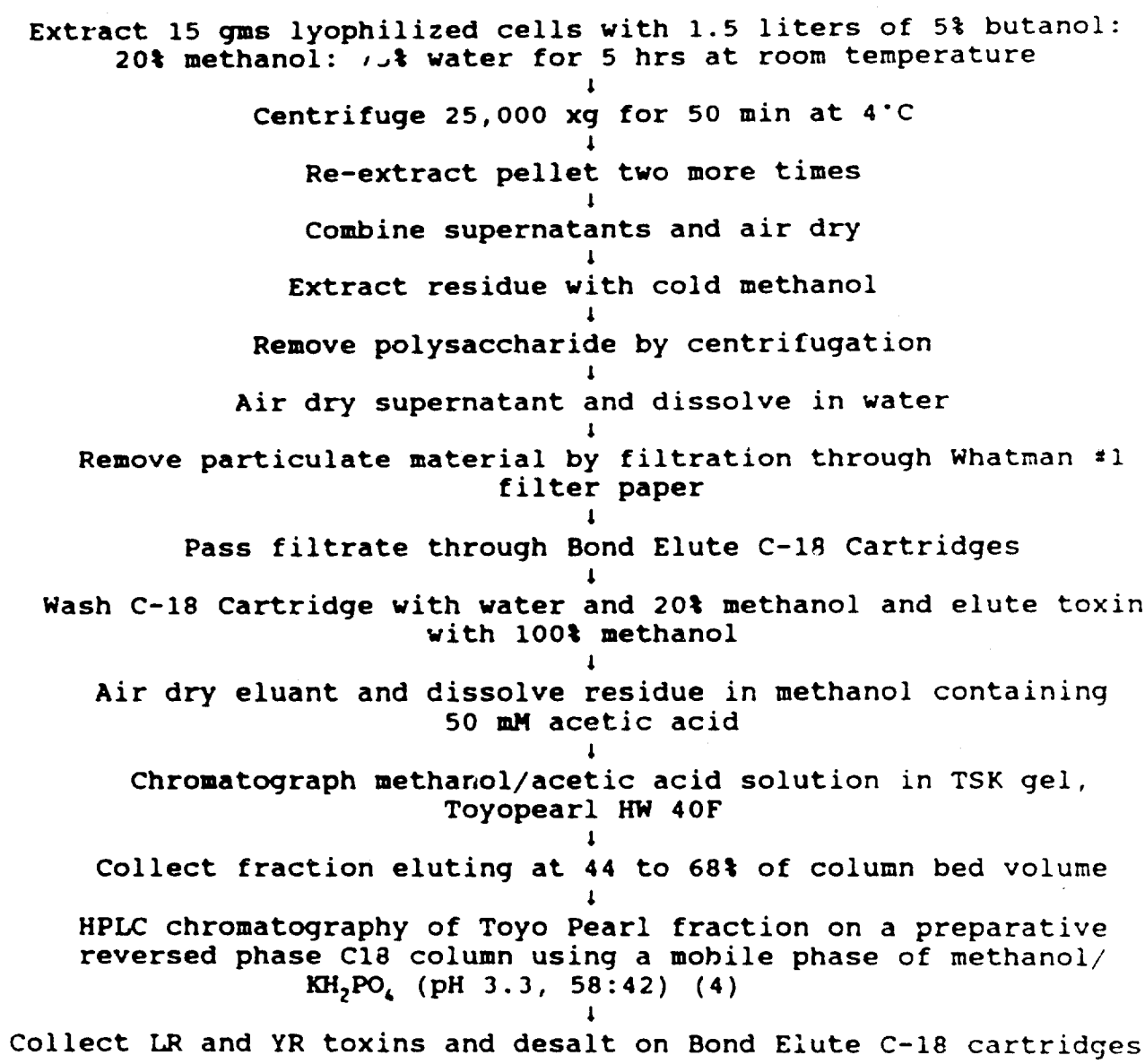
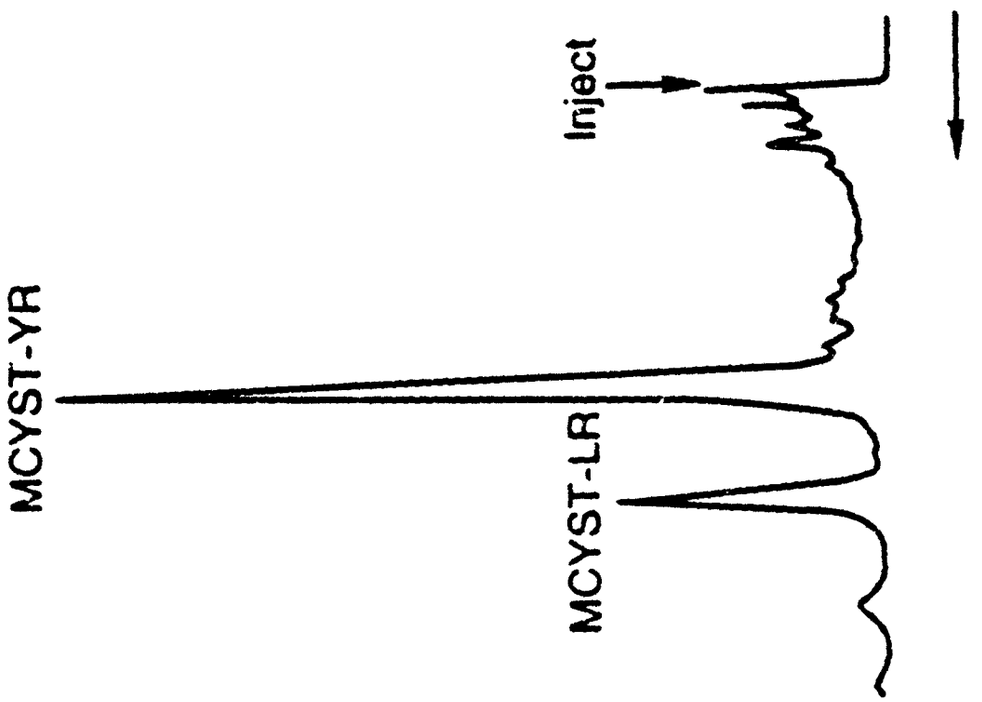


Figure 7. HPLC profile of MCYST-LR and YR produced by the Japanese strain of *M. aeruginosa* M-228.



1. Field Sample Testing and Algal Strain Isolation; b) storage and preservation of field and culture strains of cyanobacteria

1. Field sample testing and algal strain isolation.

The laboratory received incoming samples of potentially toxic algae from various sources, including public water systems, environmental health agencies, and other university-associated and independent parties. Some of these field collections were samples of algal blooms, with a high density of biomass and cells suspected of being toxigenic; other field collections were less concentrated samples of water from various survey points to monitor the presence of potentially toxic algae.

Once collected, the samples were sent via overnight delivery to the laboratory. When possible, samples were collected using a standard sample kit prepared by this laboratory and mailed prior to collection to the corresponding agency. These kits included the following: 1) two 500 mL plastic screw-cap bottles to collect adequate sample for lyophilization and toxicity testing, 2) two 25 mL screw-cap culture tubes with 10 mL BG-11 culture medium to enhance survival of algae present; 3) two 25 mL screw-cap culture tubes with 10 mL of Lugol's preservative to preserve samples for microscopic examination and identification in the event the living material is altered; 4) two empty 25 mL screw-cap culture tubes to collect sample for strain isolation; and 5) Blue Ice to keep the sample cool during return shipment.

Upon receipt, these kits were immediately processed. The contents of all containers were microscopically examined to confirm the initial report and to note the differences, if any, among the living, preserved and media-enriched samples. The large living samples were lyophilized and the media-enriched tubes were placed in an incubator. The small living samples were refrigerated until the algal strain isolations were performed, within 72 hours. The preserved samples were microscopically examined for identification of the genera, and if possible, the species present.

Isolation of the likely toxigenic algal strains in the samples was initiated once the toxicity of the parent material was confirmed by mouse intraperitoneal bioassay. These isolations were performed utilizing two methods, core isolates and drop isolates. Isolation by cores involved the following steps: 1) dilution of the field sample, usually 1:10 or 1:100; 2) inoculation of 1.5% soft agar plates (mixed with BF-11, ASM-1 or Z-8 nutrient media before cooling) with 0.5 mL of sample dilution; 3) sealing of plates and storage in an incubator for 24-72 hours; 4) identification and marking of individual filaments or colonies on the plates by use of an inverted microscope; 5) isolation of the core of soft agar containing the colony or filament by suction-drawing into a fine-tipped sterile pipette; and 6) inoculation of the core into a small culture tube with 2 mL of sterile BF-11, ASM-1 or Z-8 media (see Table 5).

Isolation by drops involved the following steps: 1) pipetting of a drop of dilute sample onto a sterile microscope slide; 2) placing of two separate nonconfluent drops of sterile media upon the same slide; 3) drawing up of a single colony or filament from the dilution sample into a flame-tapered fine tipped pipette; 4) inoculation of the colony or filament into a drop of media; 5) successive transfer of the single filament or colony to the third drop of media; and 6) inoculation of the single filament or colony from the third drop into a small culture tube with 2 mL of either BF-11, ASM-1 or Z-8 media.

Once the single filament or colony tubes were inoculated by either core or drop isolation, they were placed in an incubator under 40-60 u mol photons $m^{-2}s^{-1}$ at 24°C. The tubes were examined at regular intervals for macroscopic evidence of growth, which if evidenced, was followed by microscopic examination, to confirm that the alga growing was the isolate of interest and not a contaminant. If the strain was growing free of contamination, it was given a name based upon this laboratory's nomenclature and then successively cultured. Once a sufficient quantity of cells is obtained, lyophilization of the cells was performed so that a mouse bioassay could confirm the toxicity or nontoxicity of the isolated strain.

Laboratory nomenclature of a strain involved the assigning of a two or three letter designation utilizing all upper case letters. The first one or two letters indicated the U.S. state or Canadian province (i.e. I for Illinois, BC for British Columbia) and the successive letter indicated the body of water, or the city nearest that body of water from which the bloom material was collected (i.e. H for Homer Lake, C for Charlie Lake). Then, as each unialgal isolate was confirmed, that isolate was given a designation incorporating the letter designation from the parent strain followed by a hyphen and a numerical suffix. Thus, a hypothetical fourth unialgal isolate from Charlie Lake, British Columbia would be designated BCC-4. If field collection has a previous strain epithet, that previous designation was used.

Table 7 summarizes the field sample data for the eight samples received: collection source, strain designation, date of collection, reason for collection, LD-50 toxicity by mouse bioassay, genera described by microscopic examination, number of core isolates and number of drop isolates.

b. Maintenance and preservation of field and culture strains of cyanobacteria.

A total of 68 strains of cyanobacteria were maintained throughout the year by transfer of the unialgal culture into fresh media at four week intervals. These cultures were maintained in duplicate in 25 mL screw-cap culture tubes incubated at 40 7 mol photons m^{-2} s^{-1} and 24°C. The strains include various toxic and non-toxic representatives of the following genera: Anabaena (35 strains), Microcystis (12), Anacystis (3), Pseudanabaena (5), Aphanizomenon (3), Lyngbya (3), Oscillatoria (3), Calothrix (1), Plectonema (1), Schizothrix (1) and Synechocystis (1). Each strain was maintained

in the medium or media among BG-11, ASM-1 or Z-8 in which growth was optimal.

Upon set-up of the liquid nitrogen cryogenic storage unit, the process of freezing all of the 68 above strains and new strains as they are isolated was initiated. As of 31 October 1988, 57 of the 68 strains, plus three strains from an earlier freeze study were stored in the unit at a maximum of -123°C (=120 K). These 60 strains were prepared for cryogenic storage April through August 1988 by the following procedure: 1) centrifugation of the sample of log-phase culture for 1-2 minutes until pellet formation; 2) removal of pellet by pipette; 3) addition of equal amount of sterile Bf-11 media to pellet; 4) drawing up of 10 uL aliquots of pellet-medium mixture by micropipette; 5) freezing of 10 uL aliquot by placing in a sterile beaker of liquid nitrogen; 6) transfer of frozen pellets by sterile tweezer into cryogenic storage vial; and 7) placement of vial in cryogenic storage unit.

Throughout the year, slants, agar blocks or tubes of various cultures were received. A sample of the laboratory strain Microcystis aeruginosa UV-027 (an Isralie strain producing MCYST-RR) has been grown on agar and will be added to the list of strains maintained and preserved.

TABLE 7. Field Samples Received: 1 October 1987 - 31 October 1988

Collection Source	Strain Designation	Collection Date	Toxicity, Mouse IP Bioassay	Algal genera present	No. of core isolates	No. of drop isolates
Star Lake, NH	SL-1*	10-7-87	nontoxic @ 500 mg/kg	Anabaena	0	0
Silver Lake, NH	NHS-	8-18-88	50 mg/kg	Microcystis aeruginosa	30	10
Sadorus, IL	IS-	7-30-88	200 mg/kg	Oscillatoria; Microcystis; Anabaena	0	0
Homer Lake, IL	IH-	8-28-88	50 mg/kg	Microcystis	60	20
Pinedale, WO	WP-	9-12-88	nontoxic @ 1500 mg/kg	diatoms, desmids, Cladophora; Oedogonium	0	0
Lake Istokpoga, FL	FI-	9-21-88	20-40 mg/kg	Microcystis aeruginosa; Anabaena sp; Gonium; Pandorina; Chlorella	30	10
Charlie Lake, British Columbia	BCC-	9-29-88	insufficient material to assay	Microcystis; unicellular green algae	10	0
		10-18-88	insufficient material to assay	Microcystis; colonial green algae	30	20
Cave Lake, ID		10-11-88	insufficient material to assay	Anabaena circinalis	20	10

6. Enzyme Kinetic Studies with the Anticholinesterase Toxin ANATOXIN-A(S) from Anabaena flos-aquae NRC-525-17.

a. Protection studies.

Anticholinesterase (anti-ChE) agents inhibit acetylcholinesterase (EC 3.1.1.7, AChE) in a reaction outlined in Scheme I:

$$EOH + IX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EOHIX \xrightarrow[HX]{k_2} EOI \xrightarrow[H_2O]{k_3} EOH + HOI$$

where EOH is AChE, IX is the inhibitor either a carbamate or an organophosphate, [EOHIX] is a reversible complex, EOI is the covalently modified enzyme, IOH and HX are the hydrolyzed inhibitor and its leaving group, respectively. The rate constants, k_1 and k_{-1} define the affinity of the inhibitor; k_2 defines the rate at which the inhibitor binds and k_3 is the hydrolysis rate constant (Main, 1980). In protection studies, the aim is to: (1) halt the formation of the enzyme-inhibitor complex and (2) by the use of appropriate agents, the characteristics necessary for binding of inhibitor may be determined. In the case of AChE, the active site is composed of two subsites, the anionic and the esteratic subsite which are surrounded by regions of hydrophobicity (Wilson and Quan, 1958; Rosenberry et al., 1974). By the use of substrate and site specific reversible inhibitors (noncovalent modifers of AChE), the two points above can be determined.

The agents chosen for this study were acetylcholine (ACh), tetramethylammonium iodide (TMA, a reversible inhibitor of the anionic site) and physostigmine (Phy, a reversibie covalent modifier of the esteratic site). AChE was incubated for two minutes with increasing concentrations of ANTX-A(S) alone or in the present of ACh, TMA or Phy (Galli et al., 1985). Percent inhibition vs ANTX-A(S) concentration curves were constructed after the incubation period except for Phy which was extensively dialyzed against 100 mM sodium phosphate buffer (pH 8) to remove the carbamate group from the esteratic site. Enzyme activity was monitored by the Ellman assay (Ellman et al., 1961) and all experiments were done in triplicate. Diisopropylfluorophosphate (DFP) was used as the control anti-ChE. Figure 8 to 12 show the results of the ACh, TMA, and Phy experiments. They show that all compounds protect the enzyme, with Phy being better than TMA at approximately equal concentrations. This suggests that ANTX-A(S) has a structural component that recognizes the domains of TMA and Phy with the nucleophilic attack directed at the esteratic site.

Reactivation.

When organophosphates react with AChE, a covalent adduct is created that cannot be hydrolyzed by the weak nucleophile, water. Depending on the enzyme source (electric eel, human erythrocyte, etc.) and the nature of the alkyl groups attached to the phosphorus atom (methyl, ethyl, isopropyl), spontaneous

reactivation may occur with the regeneration of the active enzyme taking hours to days (Reiner, 1971). Wilson (see Main, 1980) first used hydroxylamine as a reactivator of diethylphosphoryl-AChE and found that the half-life to full enzyme activity could be decreased by a factor of 500 by the use of hydroxylamine. Wilson (1959) synthesized pyridine-2-aldoxime methiodide (2-PAM) based on studies of molecular complimentarity between the enzyme, substrates and inhibitors. Since then, other reactivators have been synthesized that vary in potency but are based, to some extent, on the structure of 2-PAM (Main, 1980). Mahmood and Carmichael (1987) provided evidence that ANTX-A(S) was an anticholinesterase and proposed that it behaved in a manner analogous to organophosphate inhibition of AChE. These experiments were initiated to determine if the adduct formed by ANTX-A(S) could be removed by the nucleophilic reactivators of phosphorylated AChE. If no reactivation is seen, two conclusions can be drawn. Some organophosphates undergo dealkylation of one of the two alkyl groups attached to the phosphorus in a reaction termed ageing (Main, 1980). When an organophosphate ages it is highly resistant to reactivation, either spontaneous or induced. The other possibility is that the agent is not acting at the esteratic site and therefore the reactivator is useless. This conclusion can be validated by the protection studies outlined above. In this study, paraoxon (an easily reactivatible organophosphate, DFP (ages at a slow rate) and ANTX-A(S) were used to inhibit electric eel AChE to approximately 95%. The test reactivators were 2-PAM and TMB4 at either 1 μM or 1 mM final concentration. AChE was incubated with either paraoxon, DFP or ANTX-A(S) for four minutes and then either 2-PAM or TMB4 was added and aliquots removed over time for 24 hours (Kenley et al., 1981, 1984). The results of this study (Fig. 13 to 16) indicate that ANTX-A(S) is resistant to reactivation as compared to either paraoxon or DFP although with 1 mM TMB4, there was a significant amount of 'spontaneous' reactivation seen with ANTX-A(S) that did not proceed farther than this initial amount.

In Vivo Protection.

Once an animal has been poisoned with an anti-ChE, the object is to relieve the symptoms produced by excessive cholinergic stimulation and to reactivate the inhibited enzyme. Many authors have shown the efficacy of the oxime reactivators and the use of carbamate pretreatment to either alleviate or subdue the symptoms of cholinesterase poisoning (Albuquerque et al., 1985; Deyi et al., 1981; Harris et al., 1984; Koelle, 1948; Lennox et al., 1985; Shiloff and Clements, 1985). This study looked at the effects of atropine sulfate and TMB4 or 2-PAM given alone or in combination on the lethality of 49 ug/kg ANTX-A(S). ICR Swiss male mice (15 to 20 grams) were given free access to food and water. The dose of ANTX-A(S) produced 100% mortality. The experimental dosing consisted of injection of ANTX-A(S) followed by a saline blank, atropine, 2-PAM, TMB4 or atropine and 2-PAM (or TMB4). 2-PAM at 35 mg/kg and 10 mg/kg atropine sulfate injected just after ANTX-A(S) lead to 60% mortality whereas 2-PAM and atropine sulfate given 30 minutes prior to ANTX-A(S)

injection lead to 100% mortality. 2-PAM or TMB4 at 14.3 mg/kg and 10 mg atropine sulfate lead to 100% mortality. Physostigmine at 0.2 mg/kg given 30 minutes prior to ANTX-A(S) lead to 40% mortality although the animals showed signs of cholinesterase poisoning with labored breathing and generalized shaking predominating (Fig. 17).

b. Receptor Effects.

Anti-ChE agents along with their inhibition of cholinesterase, also have direct effects on predominantly nicotinic but also on muscarinic receptors. As a class, the carbamates have the strongest interactions with the nicotinic acetylcholine receptor-ion channel complex and the organophosphates producing similar effects (Albuquerque et al., 1984; Fredriksson and Tibbling, 1959; Pascuzzo et al., 1981; Yamada et al., 1982). To probe the nicotinic effects of ANTX-A(S), the frog rectus abdominus muscle preparation was used. First, determination of lethality of ANTX-A(S) in the frog was determined. ANTX-A(S) was administered by stomach tube, percutaneously, and by injection into the dorsal lymph sac or intraperitoneally. Only intraperitoneal injection proved lethal and the LD_{50} was determined to be 281.25 µg/kg (1 µM). In the isolated muscle experiments, dose-response curves were generated with ANTX-A (a potent nicotinic agonist) before and after the muscle was exposed to ANTX-A(S) for 10 minutes. ANTX-A(S) at concentrations of 1 and 10 uM (Fig. 18 and 19) did not stimulate the muscle to contract nor block the ANTX-A induced contraction. It is concluded that ANTX-A(S) devoid of nicotinic activity.

To probe for muscarinic activity, two experimental procedures were used. To determine functional effects, the denervated guinea pig ileum was used and rat forebrain homogenate was used to look at receptor binding using the technique of Yamamura and Snyder (1974). Segments of ileum were denervated by refrigeration under a nitrogen atmosphere in 10% glucose-Tyrode's solution for 24 hours. The segments were suspended in a 20 ml organ bath, warmed to 37°C and contraction height vs. bethanechol (muscarinic agonist that is not hydrolyzed by AChE) concentration curves generated. After the control curves, the ileal segment was subjected to either a ten minutes incubation with ANTX-A(S) followed by repeated washings for ten minutes or being exposed to ANTX-A(S) just prior to being exposed to bethanechol and the bethanechol dose-response curve repeated. Figure 20 and 21 shows the dose-dependent decrease in bethanechol elicited contractions after treatment with ANTX-A(S). The results show a functional antagonism of the muscarinic receptor although the mechanism is unknown. Figure 22 shows the effects of DFP on the ileum. Replotting the data as the recipercols analogous to the Lineweaver-Burke transformation indicates that ANTX-A(S) does not compete with bethanechol at the muscarinic receptor. Pretreating the ileal segment with atropine (10^{-11}M) (Fig. 23) before exposure to ANTX-A(S) eliminates the ANTX-A(S) effect. Pretreating the muscle first with ANTX-A(S) and then atropine (Fig. 24) shows a slight shift to the right of the atropine curve suggesting a

synergistic action, although the significance of the result is unknown at this time.

Examining ANTX-A(S) effect at the receptor level shows that coincubating ANTX-A(S) with QNB produces no change in QNB binding at the muscarinic receptor but preincubating ANTX-A(S) with the receptors for 5 minutes and then incubating with QNB shows an ANTX-A(S) dose dependent decrease in QNB binding. This points again to a 'non-competitive' mechanism by which ANTX-A(S) binds to muscarinic receptors.

Fukuda et al. (1988) have shown that potassium channels are linked to muscarinic receptors and are activated upon agonist binding. In the frog semitendinosus muscle in a chloride free solution, membrane potential is generated by potassium channel activity. ANTX-A(S) exposed to the muscle while recording membrane potential with an intracellular microelectrode did not change the membrane potential or interfere with the potassium channel as determined by subsequent depolarization of the muscle by barium ions (potassium channel blocker).

pH Stability of ANTX-A(S).

In the isolation, purification and analysis of ANTX-A(S) various pH levels are reached and the effect of these levels is unknown on the inhibition of degradation of ANTX-A(S). Equal amounts of ANTX-A(S) were put into three tubes of 100 mM sodium phosphate buffer: pH 3, pH 7 and pH 12. Inhibition was determined by incubating ANTX-A(S) and AChE for two minutes and determining enzyme activity with the Ellman assay. Percent Inhibition (determined in triplicate) was determined once a day for five days and at 30 days. At pH 3, ANTX-A(S) is stable throughout the whole time period, at pH 7 there is approximately 7% decrease in activity and at pH 12 within the time frame of the day-one assay (≈30 seconds) there is 90 to 95% decrease in the inhibition as compared to pH 3. This level remained constant throughout the experiment (Fig. 25).

c. Summary.

ANTX-A(S) is an active site directed anticholinesterase that is resistant to in vivo and in vitro reactivation. ANTX-A(S) has no activity at nicotinic receptors and appears to be a non-competitive antagonist of muscarinic receptors.

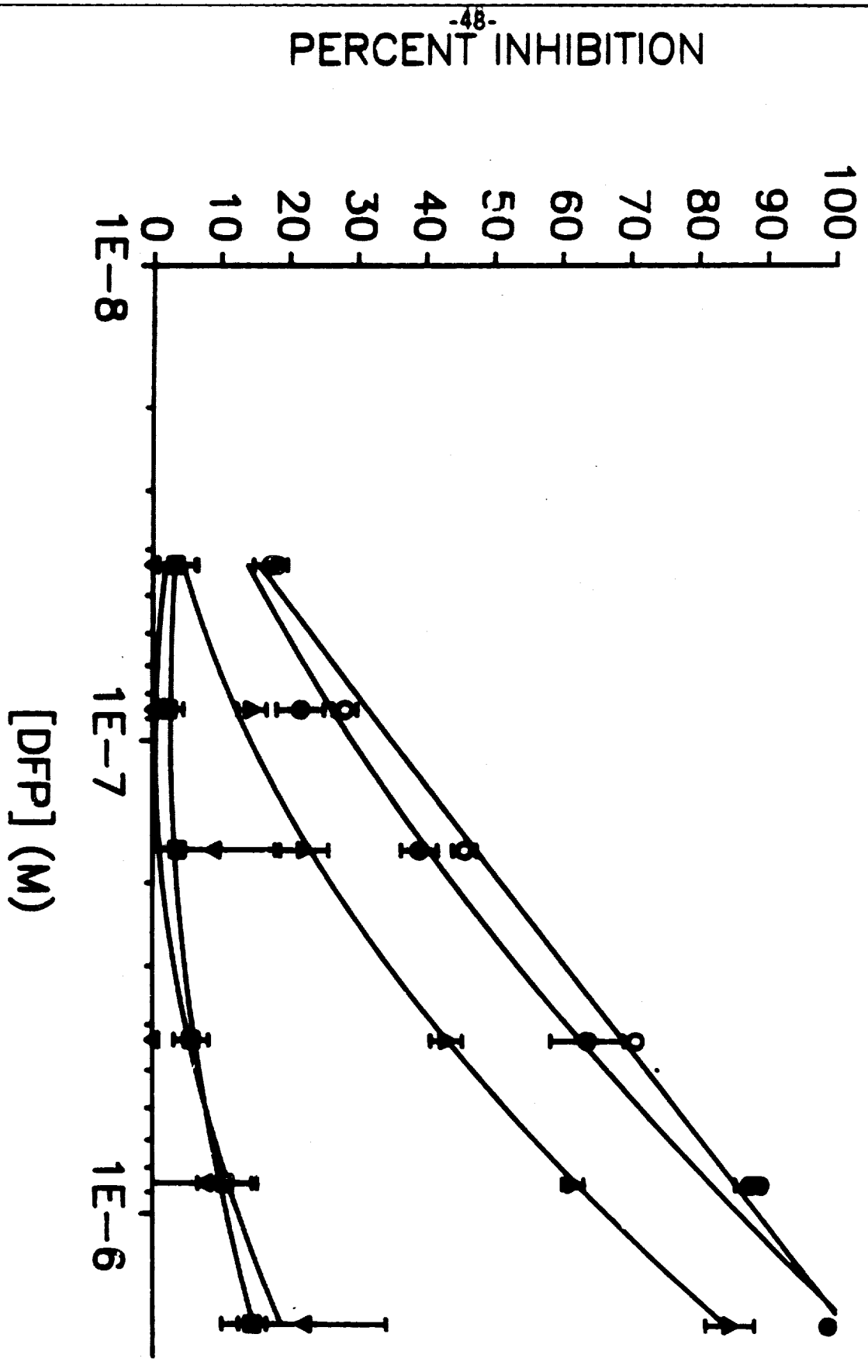


Fig. 8. ACh protection of electric eel AChE from DFP-symbols:
o - control DFP inhibition
● - 1 μM ACh coincubated with DFP
▲ - 10 μM ACh coincubated with DFP
■ - 100 μM ACh coincubated with DFP
▼ - 10 mM ACh coincubated with DFP

DFP and ACh or buffer was incubated for 2 minutes with AChE before activity was measured. The values are the mean ± SEM of three experiments.

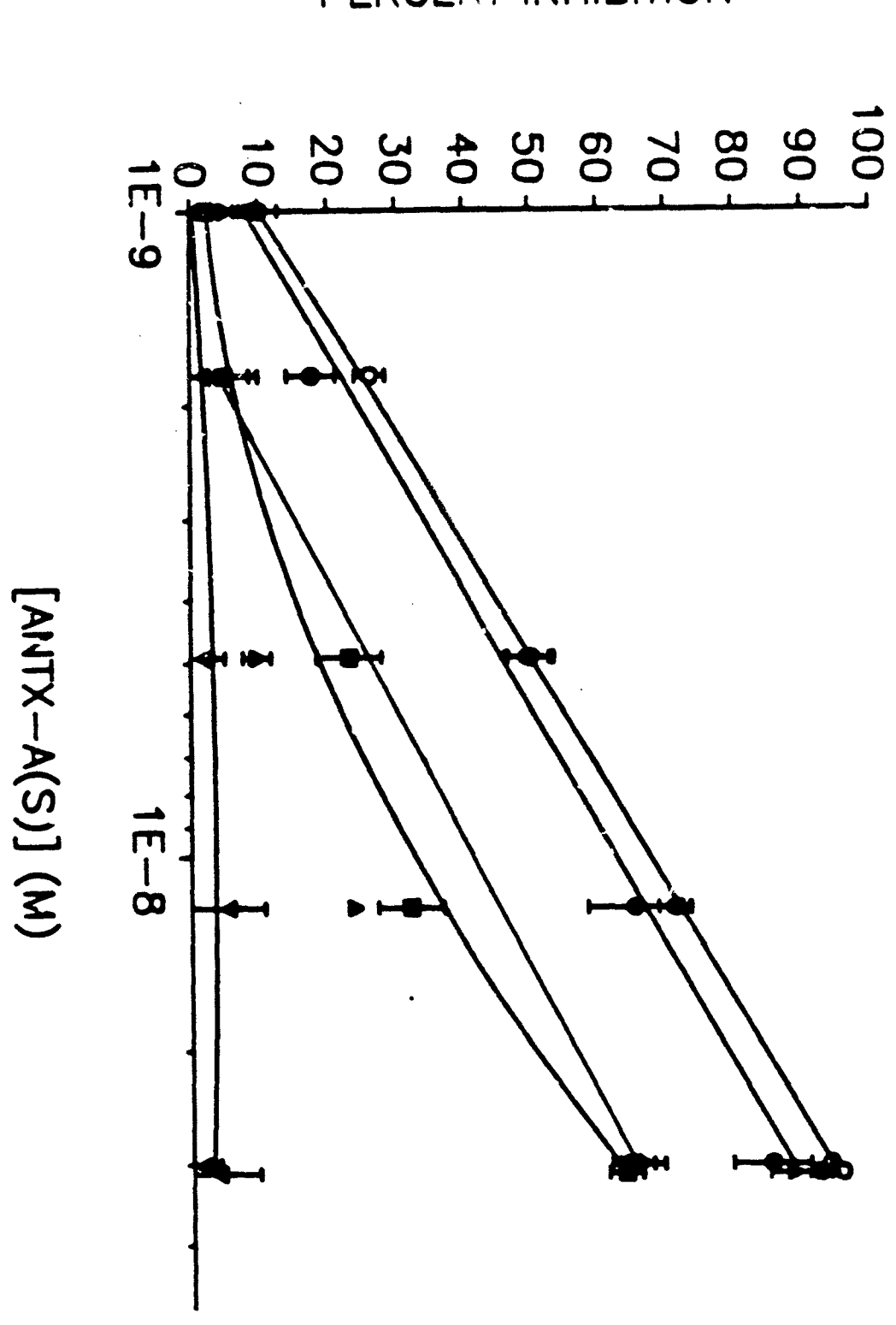


Fig. 9. ACh protection of electric eel AChE from ANTX-A(S) symbols and conditions same as Fig. 8.

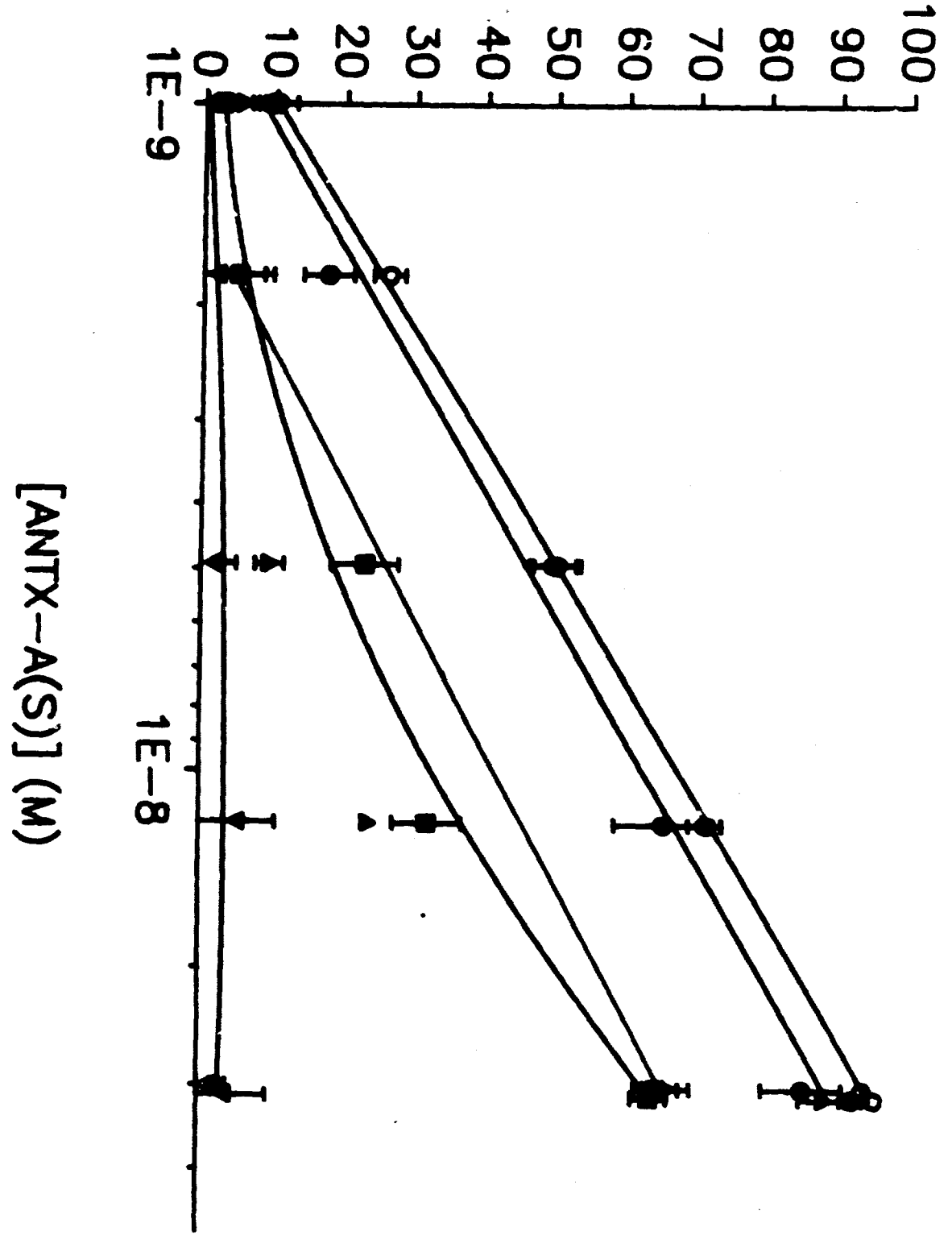


Fig. 9. ACh protection of electric eel AChE from ANTX-A(S) symbols and conditions same as Fig. 8.

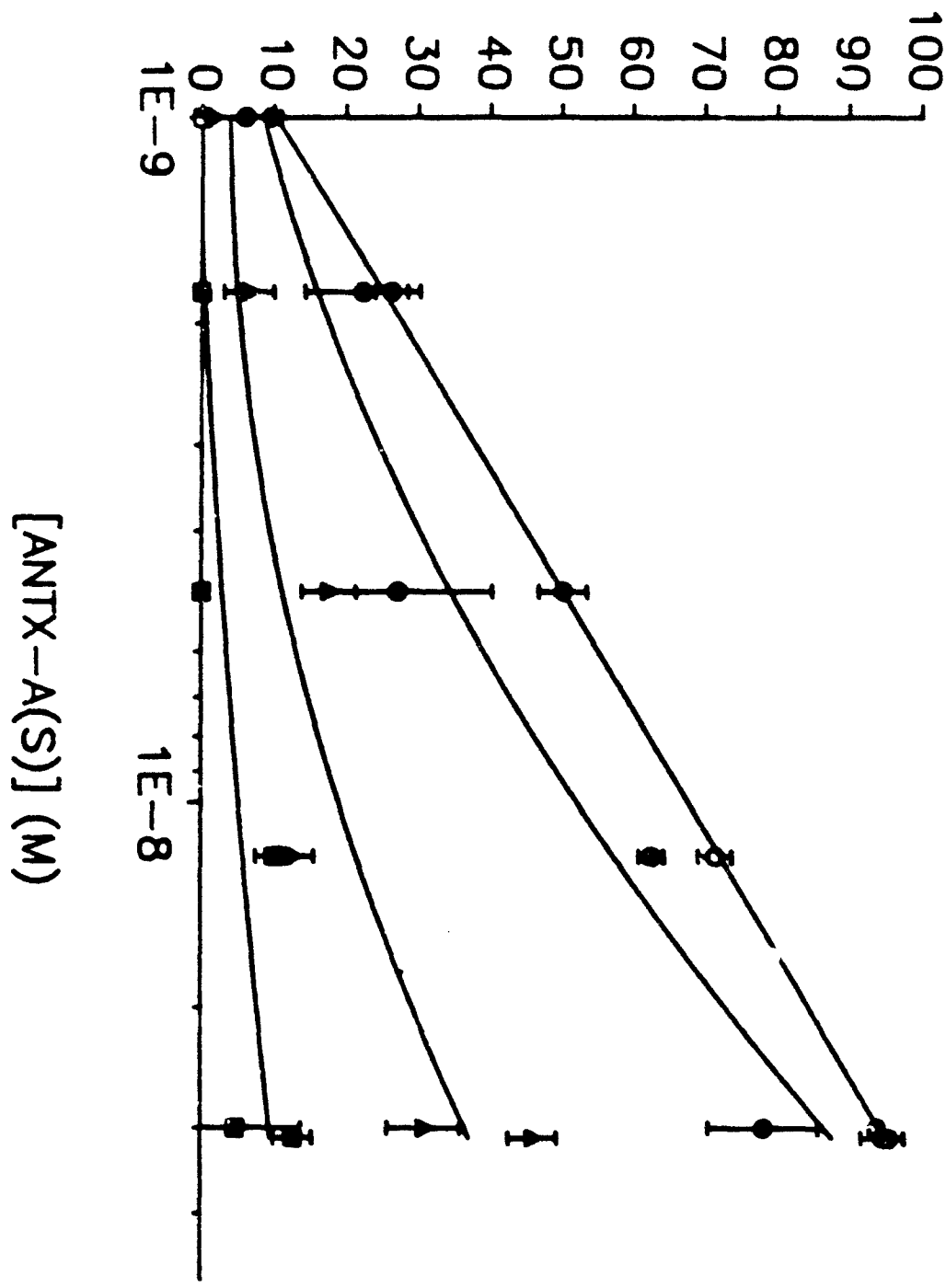
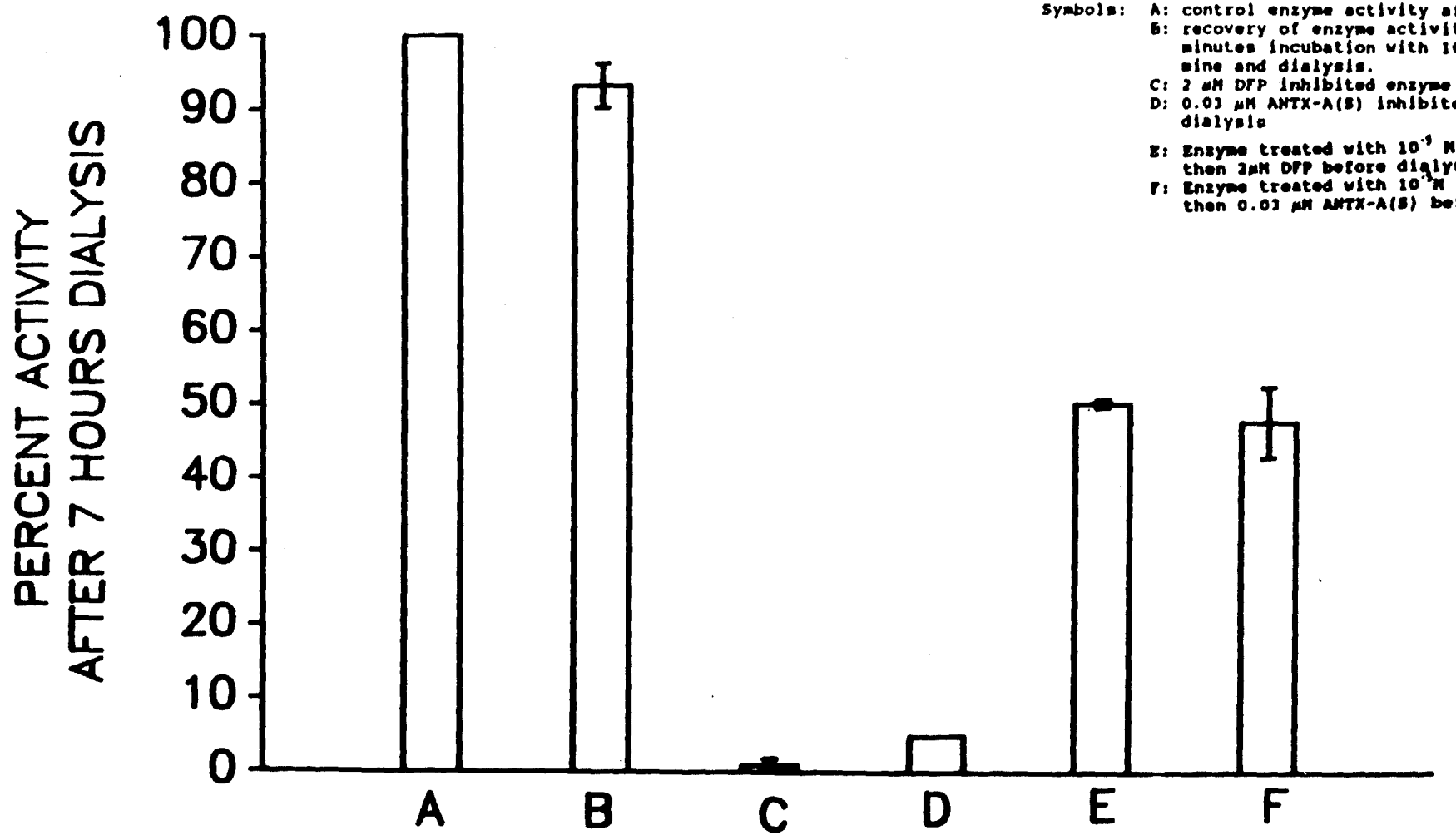


Fig. 11. THA protection of electric eel AChE from ANTX-A(S) symbols are the same as in Fig. 10.

Fig. 12. Physostigmine protection of electric eel AChE from DFP and Antx-a(s).

Enzyme was incubated with buffer or 10^{-3}M Physostigmine for 30 minutes then treated for 2 minutes with buffer, 2 μM DFP or 0.03 μM ANTX-A(S) then dialyzed against 100 mM sodium phosphate buffer, pH 8 for 7 hours before activity was determined.

Symbols:
- A: control enzyme activity after dialysis
- B: recovery of enzyme activity after 30 minutes incubation with 10^{-3}M Physostigmine and dialysis.
- C: 2 μM DFP inhibited enzyme after dialysis
- D: 0.03 μM ANTX-A(S) inhibited enzyme after dialysis
- E: Enzyme treated with 10^{-3} M Physostigmine then 2μM DFP before dialysis
- F: Enzyme treated with 10^{-3}M Physostigmine then 0.03 μM ANTX-A(S) before dialysis



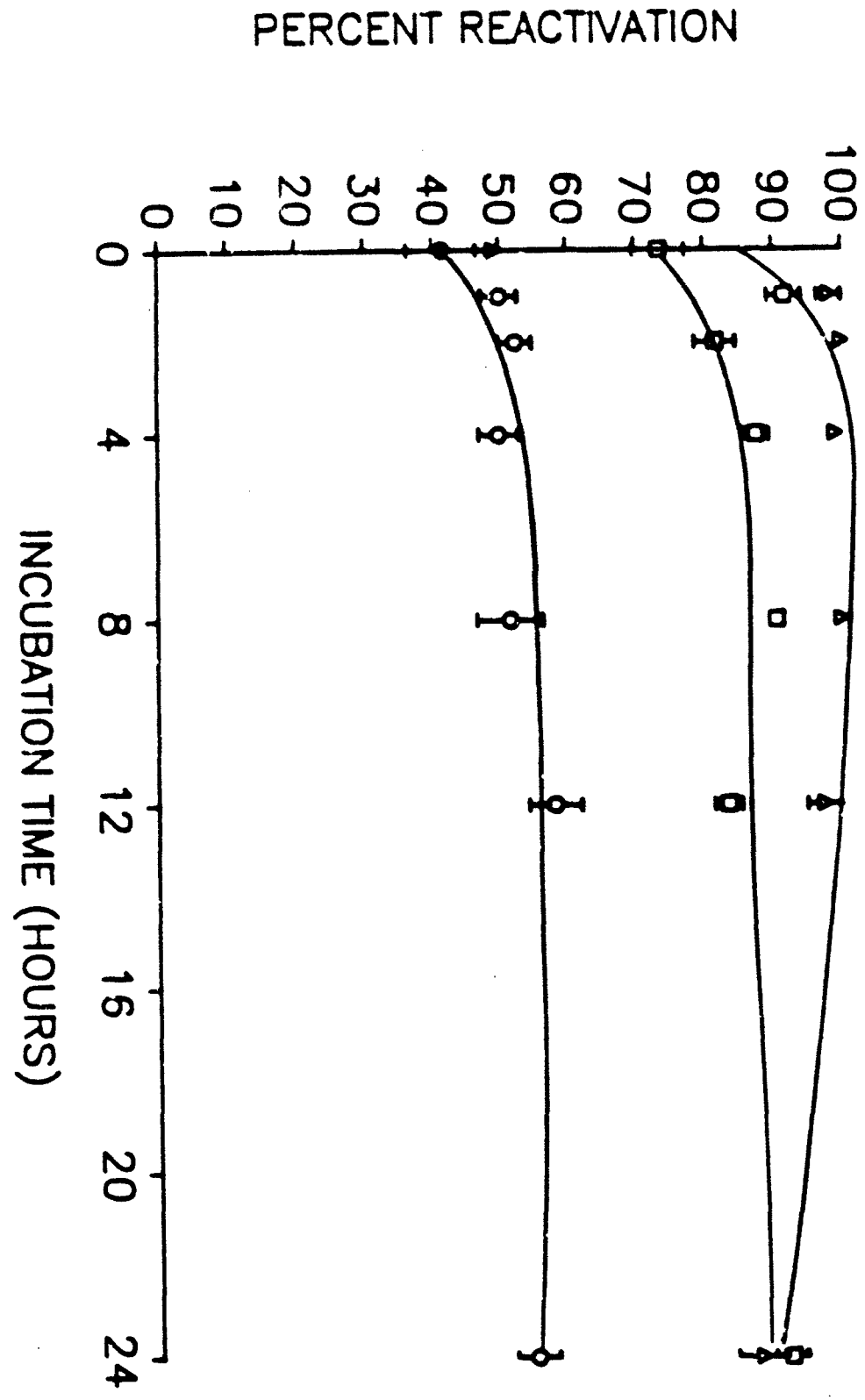


Fig. 13. Analysis of Reactivation Potential of 1 mM 2-PAM against DFP (□): Paraoxon (△) and AMTX-A(S) (○).

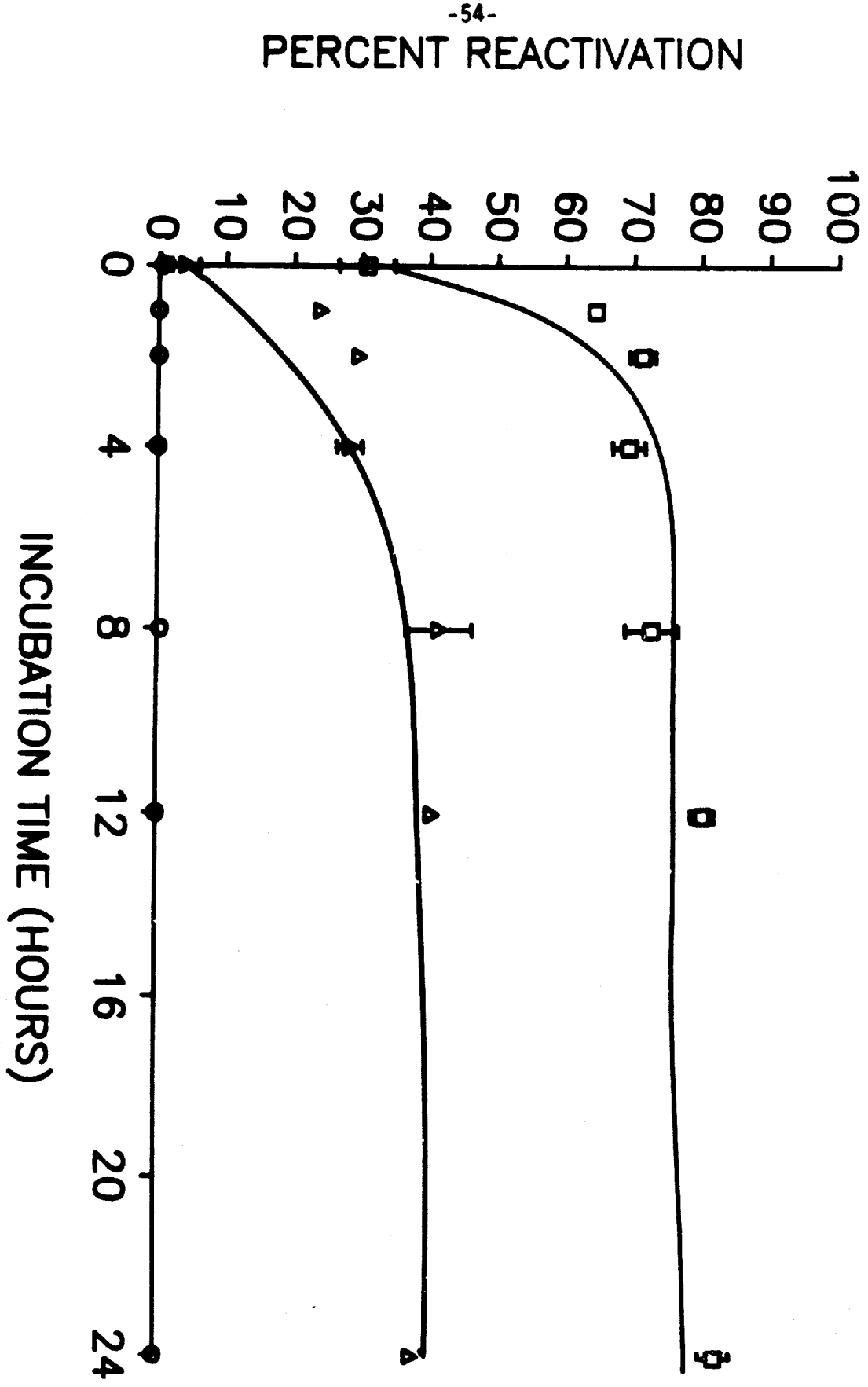


Fig. 14. Analysis of Reactivation Potential of 1 μM 2-PAM against DFP (□): Paraoxon (▲) and AMTX-A(S) (O).

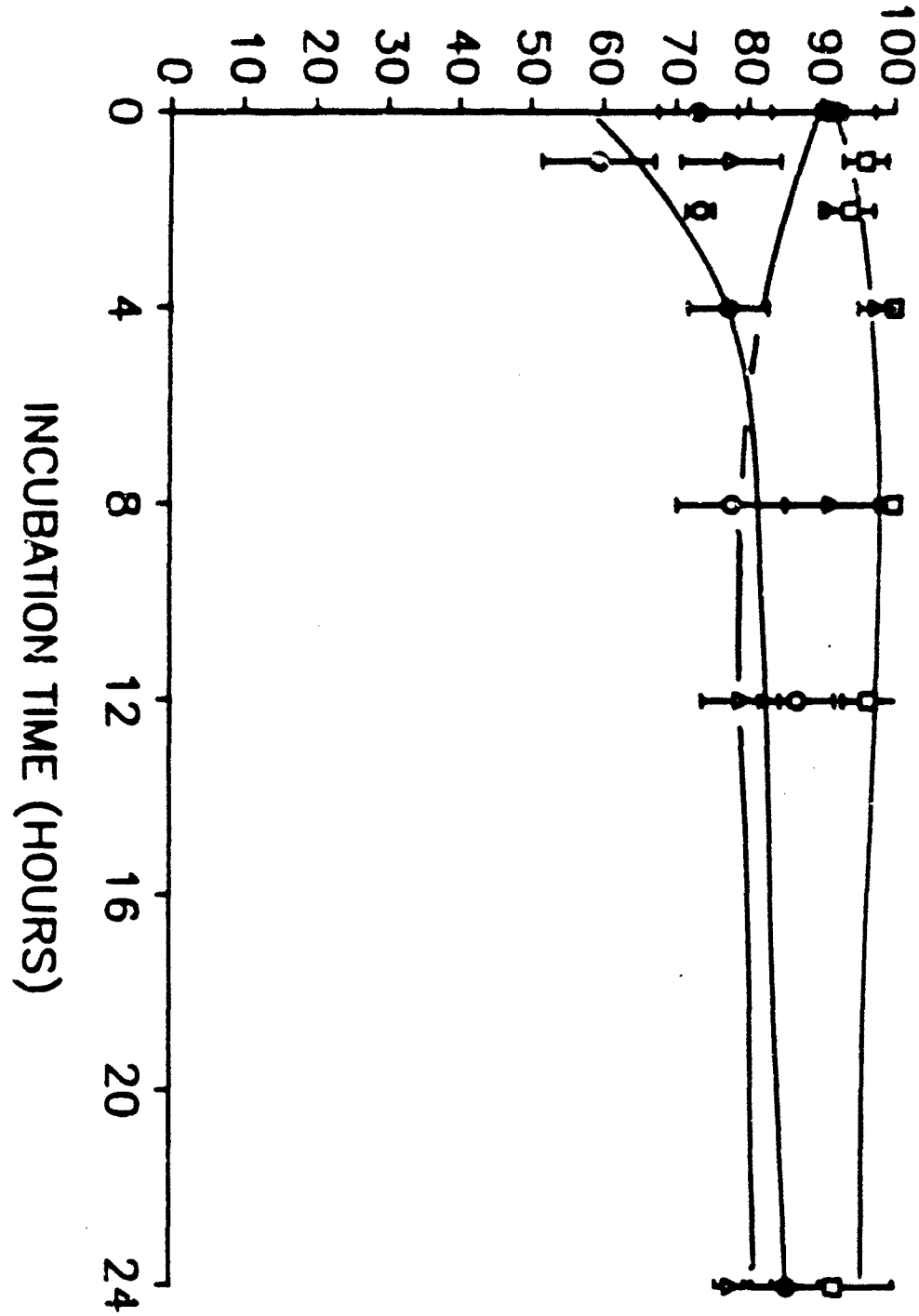


Fig. 13. Analysis of Reactivation Potential of 1 mM TMB4 against OFP (□): Paraoxon (▲) and AmTX-A(S) (○).

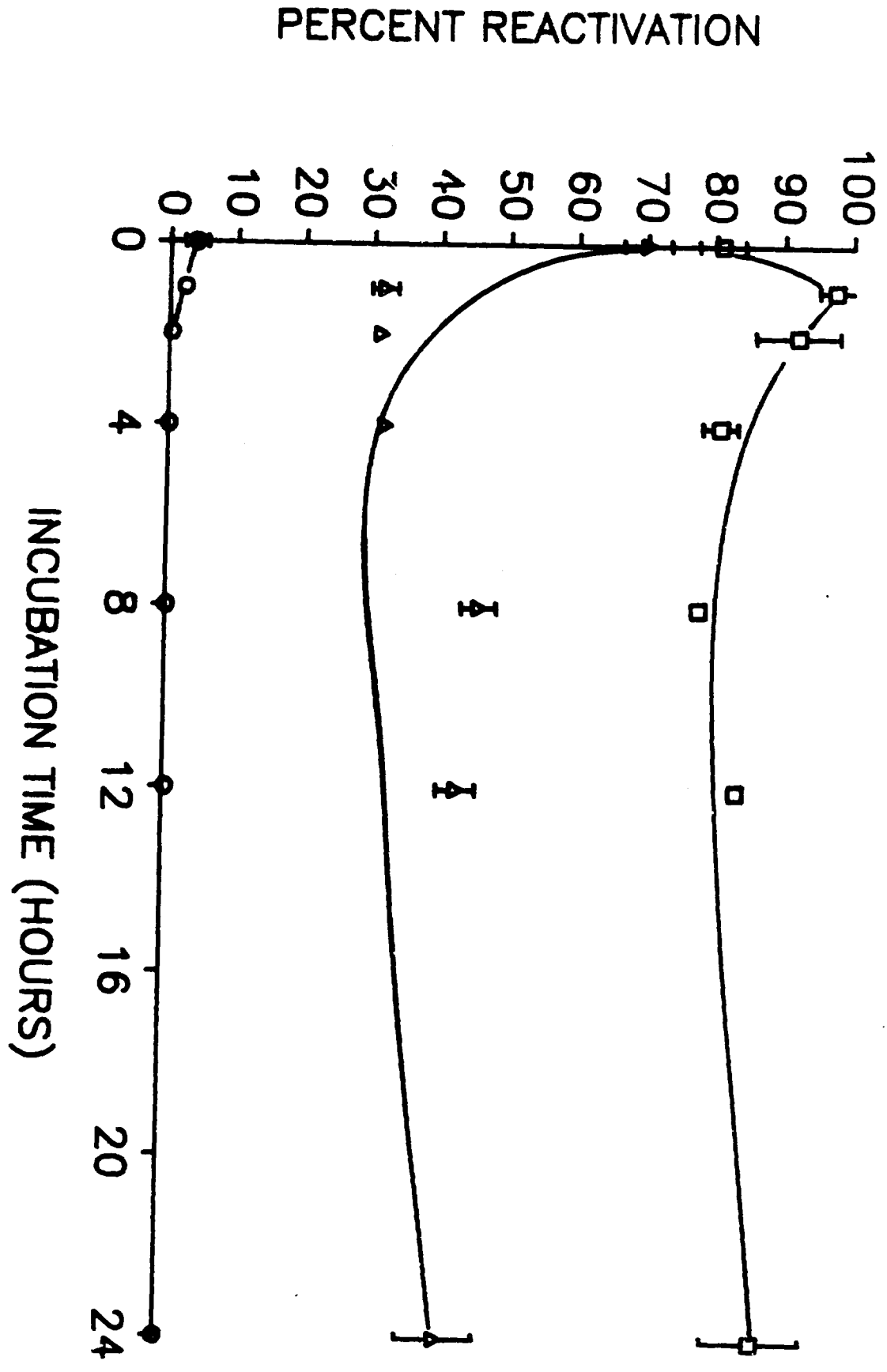


Fig. 15. Analysis of reaction potential of 1 μM TMB4 against DFP (□); Paraoxon (▲) and Antx-a(s) (O).

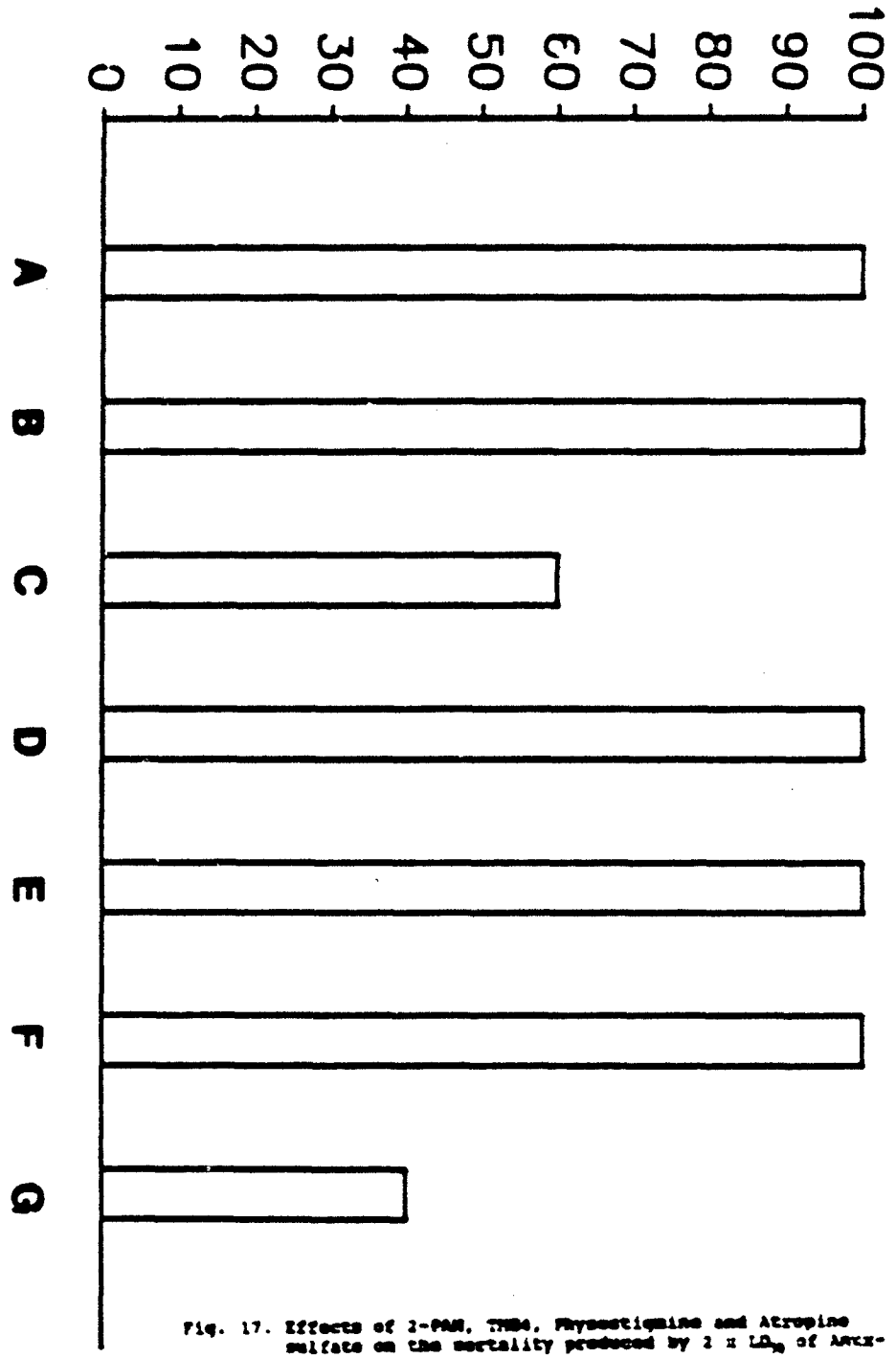


Fig. 17. Effects of 2-PAM, TMB4, Physostigmine and Atropine sulfate on the mortality produced by 2 x LD_{50} of Anvx-a(s).

A - AHTX-A(S) at 2 x LD_{50}
B - 14.3 mg/kg 2-PAM and 10 mg/kg atropine sulfate
C - 35 mg/kg 2-PAM and 10 mg/kg atropine sulfate
D - 35.3 mg/kg 2-PAM and 10 mg/kg atropine sulfate given 30 minutes prior to AHTX-A(S)
E - 14.3 mg/kg TMB4 and 10 mg/kg atropine sulfate
F - 35 mg/kg TMB4 and 1u mg/kg atropine sulfate
G - 0.2 mg/kg Physostigmine and 10 mg/kg atropine sulfate given 30 minutes prior to AHTX-A(S).

5 animals/group.

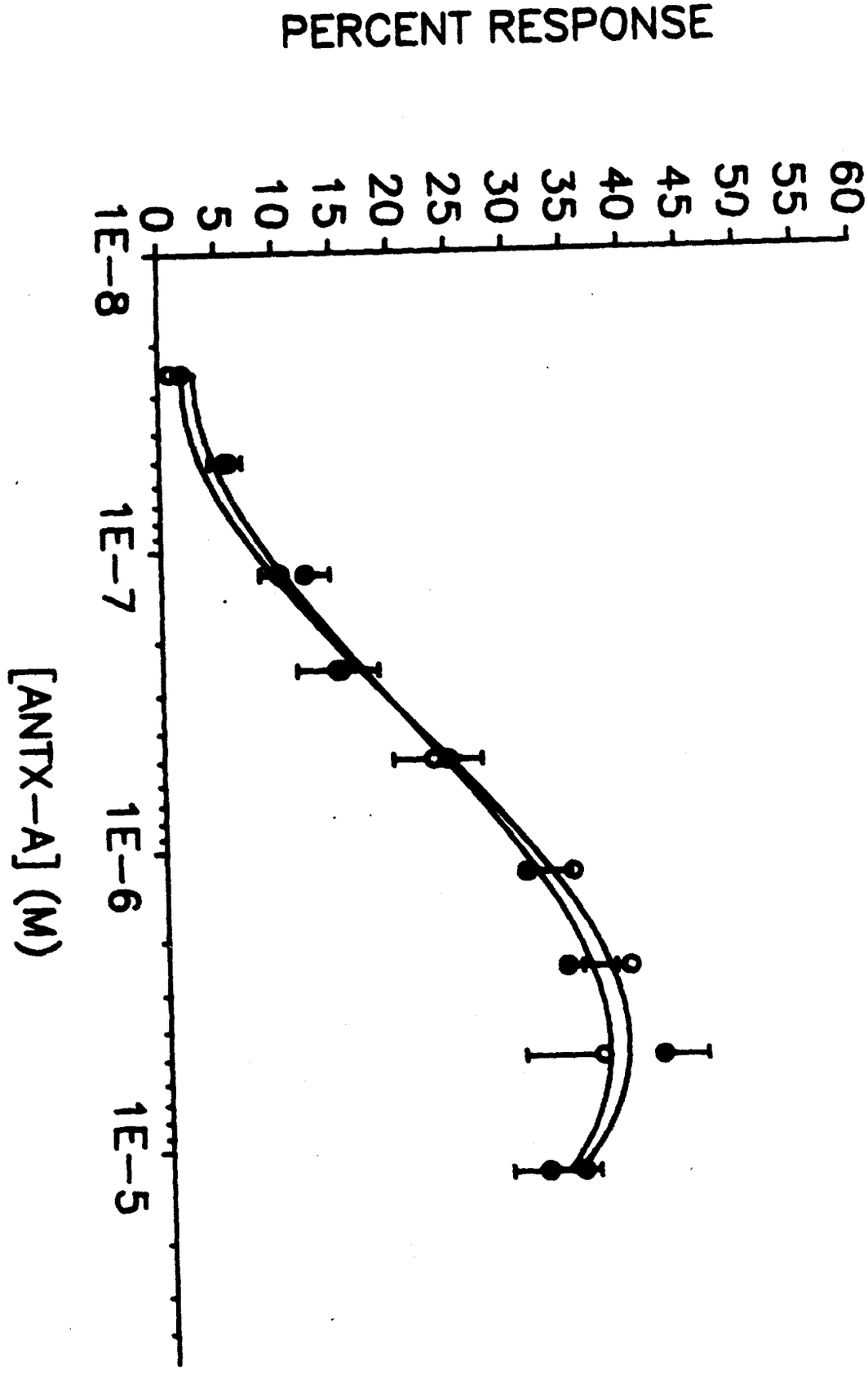


Fig. 18. Probe of nicotinic activity of ANTX-A(S) on isolated frog rectus abdominus muscle. Symbols: (O) - ANTX-A dose response curve, (●) muscle was pretreated with 1 μM (6.7 μg/20 mls) of antx-a(s) for 10 minutes then washed; ANTX-A dose response curve repeated. Results are the mean ± SEM of eight experiments.

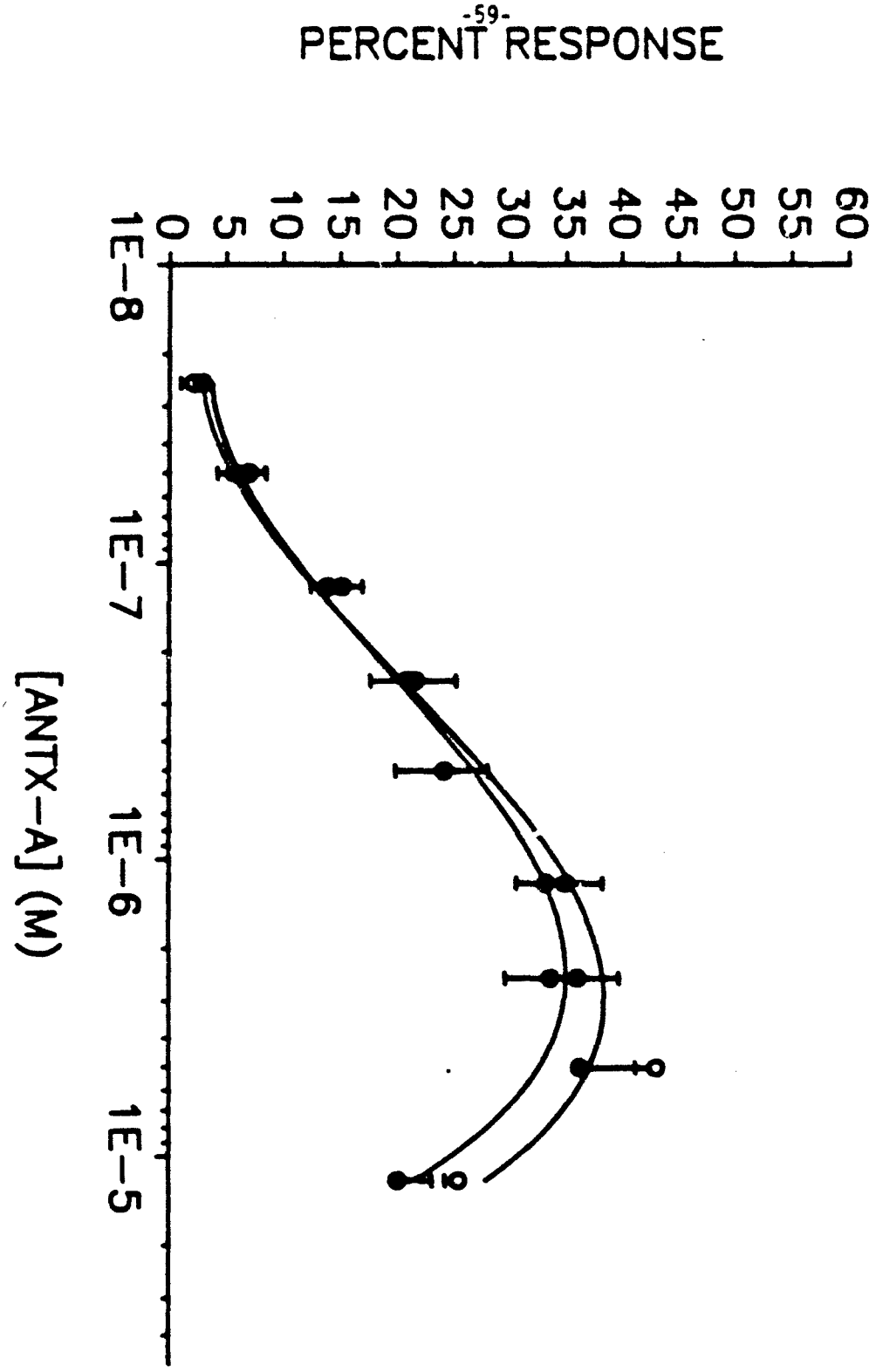


Fig. 19. Probe of nicotinic activity of ANTX-A(S) on isolated frog rectus abdominus muscle. 10 μM (67 μg/20 ml) ANTX-A(S) used, symbols the same as in Fig. 18.

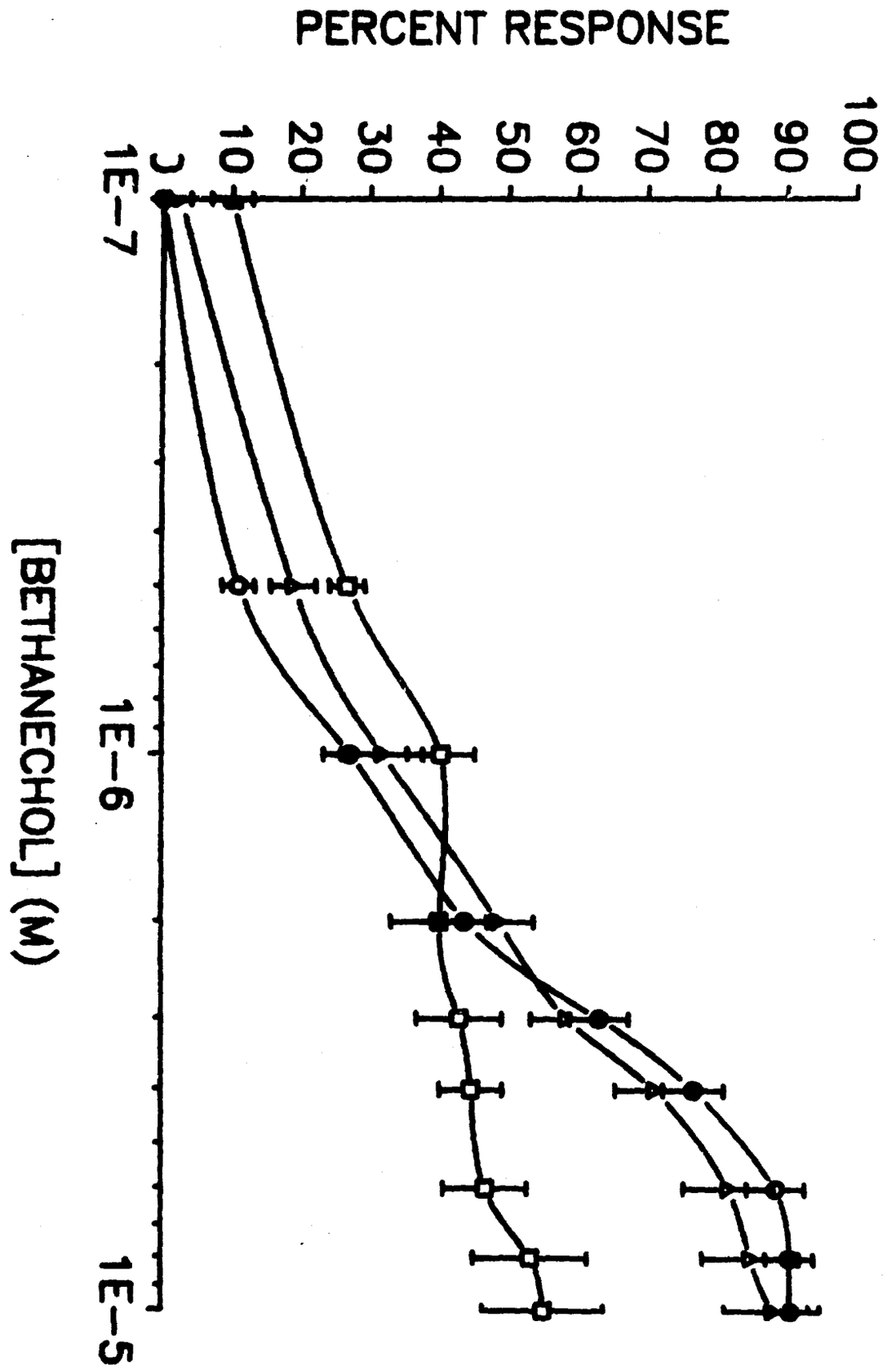


Fig. 20. Probe of muscarinic activity ANTX-A(S) on isolated, denervated guinea pig ileum. Symbols: (O) control bethanechol dose-response curve; (▲) muscle pretreated with 0.1 μM (0.67 μg/20 mls) ANTX-A(S) for 10 minutes before each bethanechol dose; (□) muscle given ANTX-A(S) just prior to bethanechol. The results are the mean ± SEM for at least eight experiments.

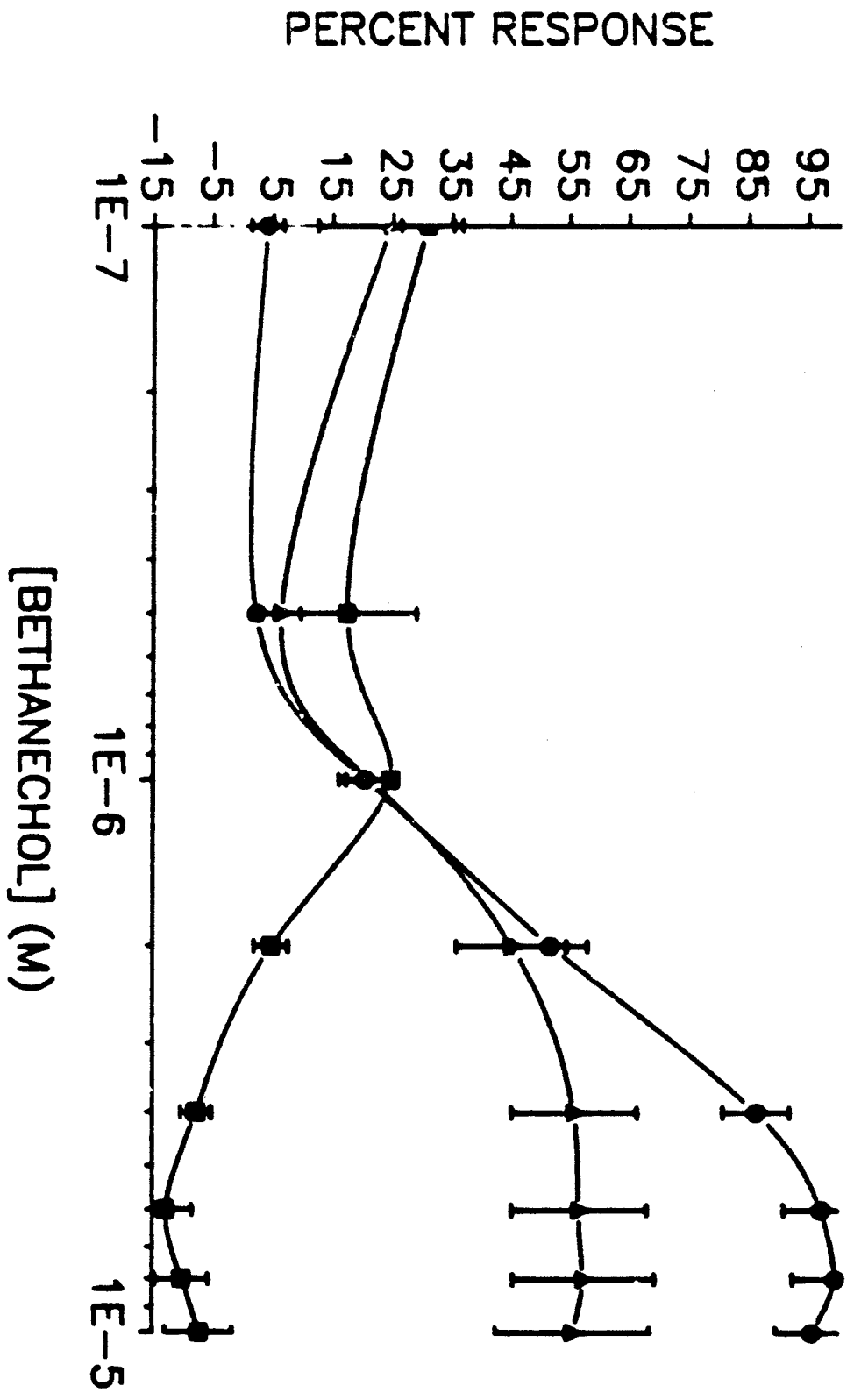


Fig. 21. Probe of muscarinic activity of AMTX-A(S) on isolated, denervated guinea pig ileum. Symbols: (●) control bethanechol dose-response curve; (▲) muscle pretreated with 1 μM (6.7 μg/20 ml) AMTX-A(S) for 10 minutes before each bethanechol dose; (○) muscle given AMTX-A(S) just prior to bethanechol. The results are the mean ± SEM for at least eight experiments.

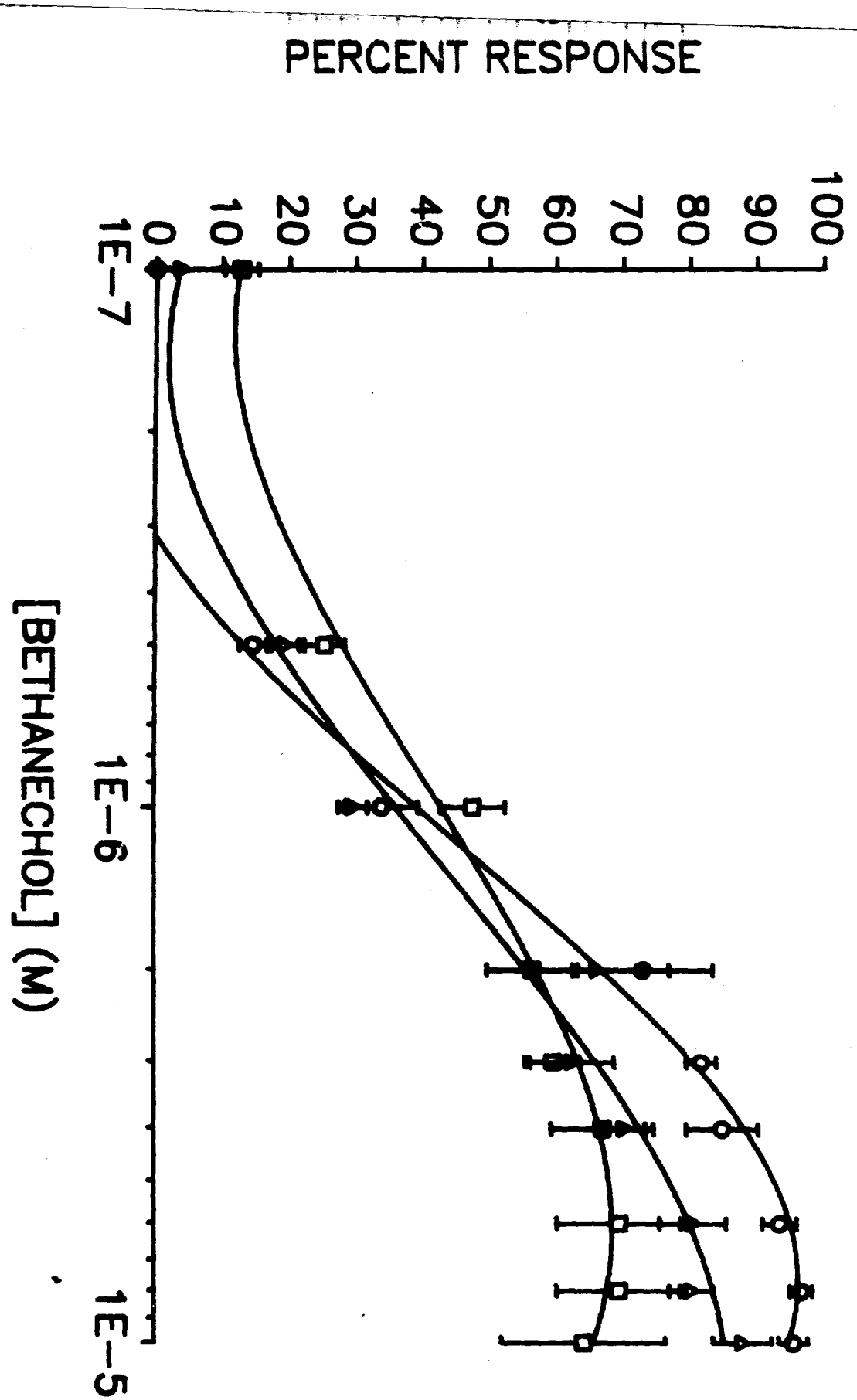


Fig. 22. Response of the isolated denervated guinea pig ileum to 6.7 ug/20mls (2uM) DFP. Symbols have the same meaning as in Fig. 21.

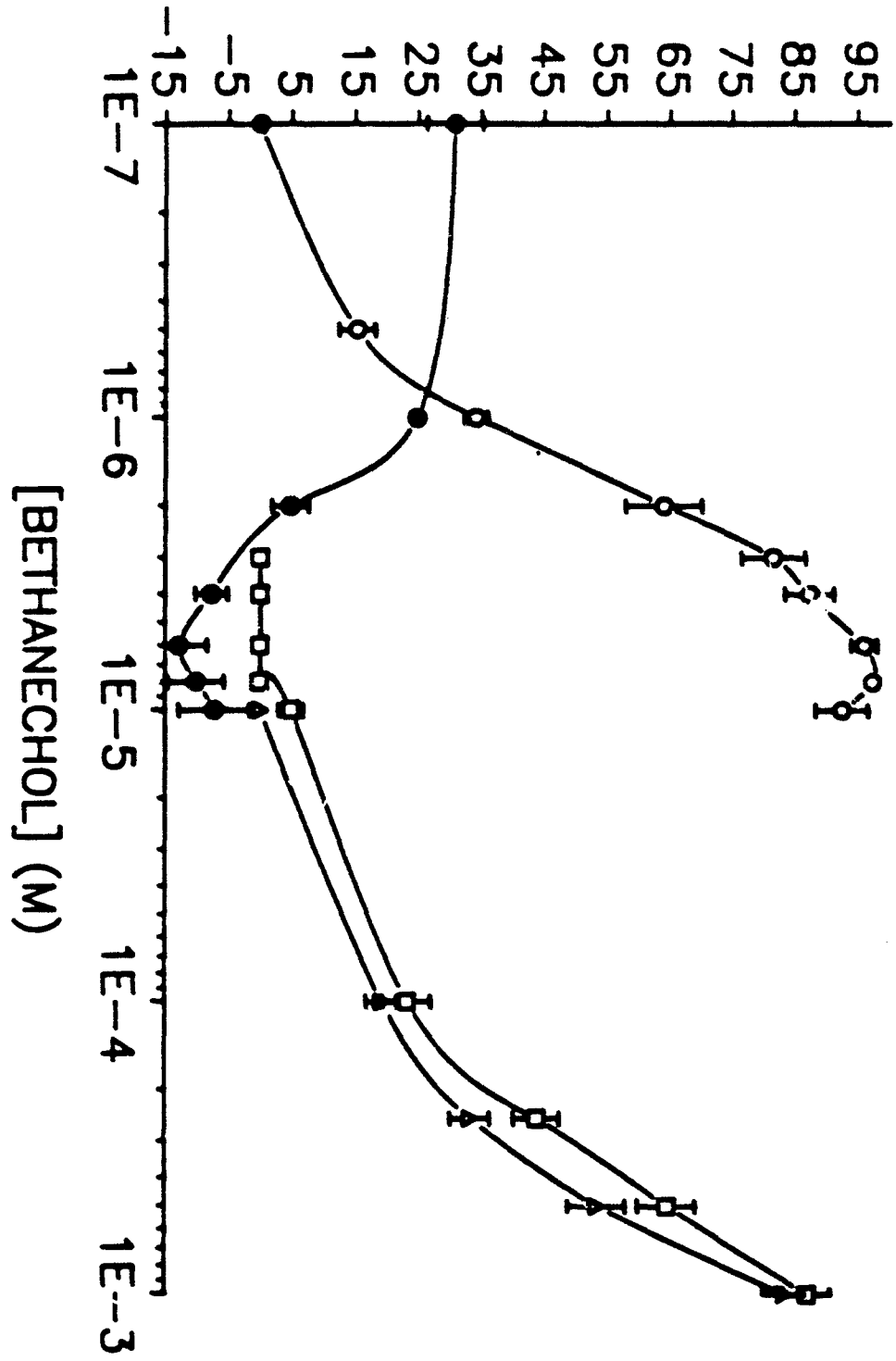


Fig. 23. The response of the guinea pig ileum to AMTX-A(S) in the presence of 10^{-11} M atropine sulfate. Symbols: (O) bethanechol dose-response curve; (●) 1 nM (6.7 ng/20 ml) AMTX-A(S) given just before each bethanechol dose; (▲) bethanechol dose-response curve in the presence of 10^{-9} M atropine sulfate; (□) AMTX-A(S) given just before each bethanechol dose in the presence of 10^{-9} M atropine sulfate. The results are the mean ± SEM of eight experiments.

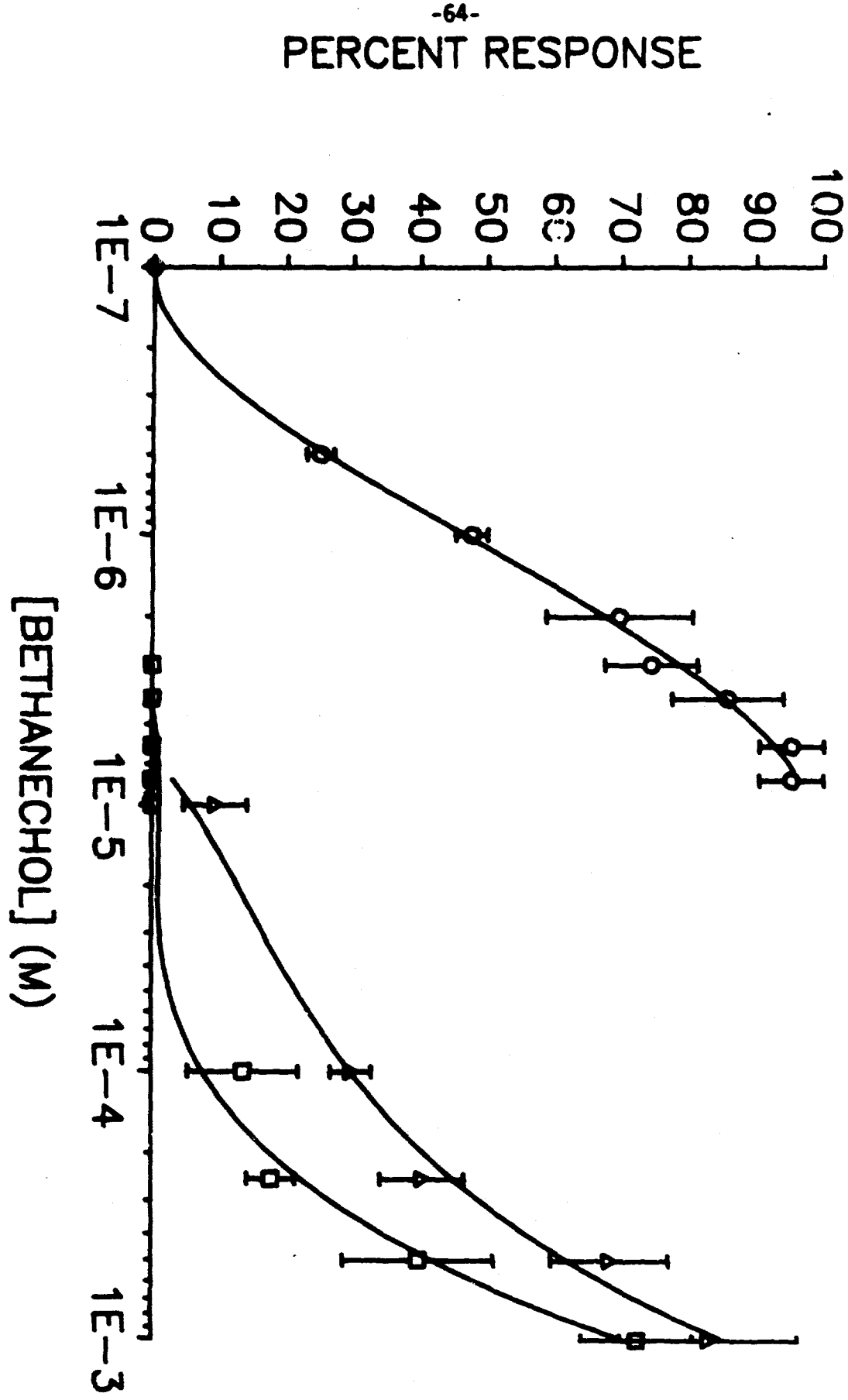


Fig. 24. The response of the guinea pig ileum to ANTX-A(S) pretreatment followed by atropine sulfate. Symbols: (O) bethanechol dose-response curve: (Δ) bethanechol dose response curve in presence of 10^{-11} M atropine sulfate: (□) the muscle exposed to 1 μM (6.7 μg/20 mls) ANTX-A(S) then 10^{-11} M atropine sulfate then bethanechol.

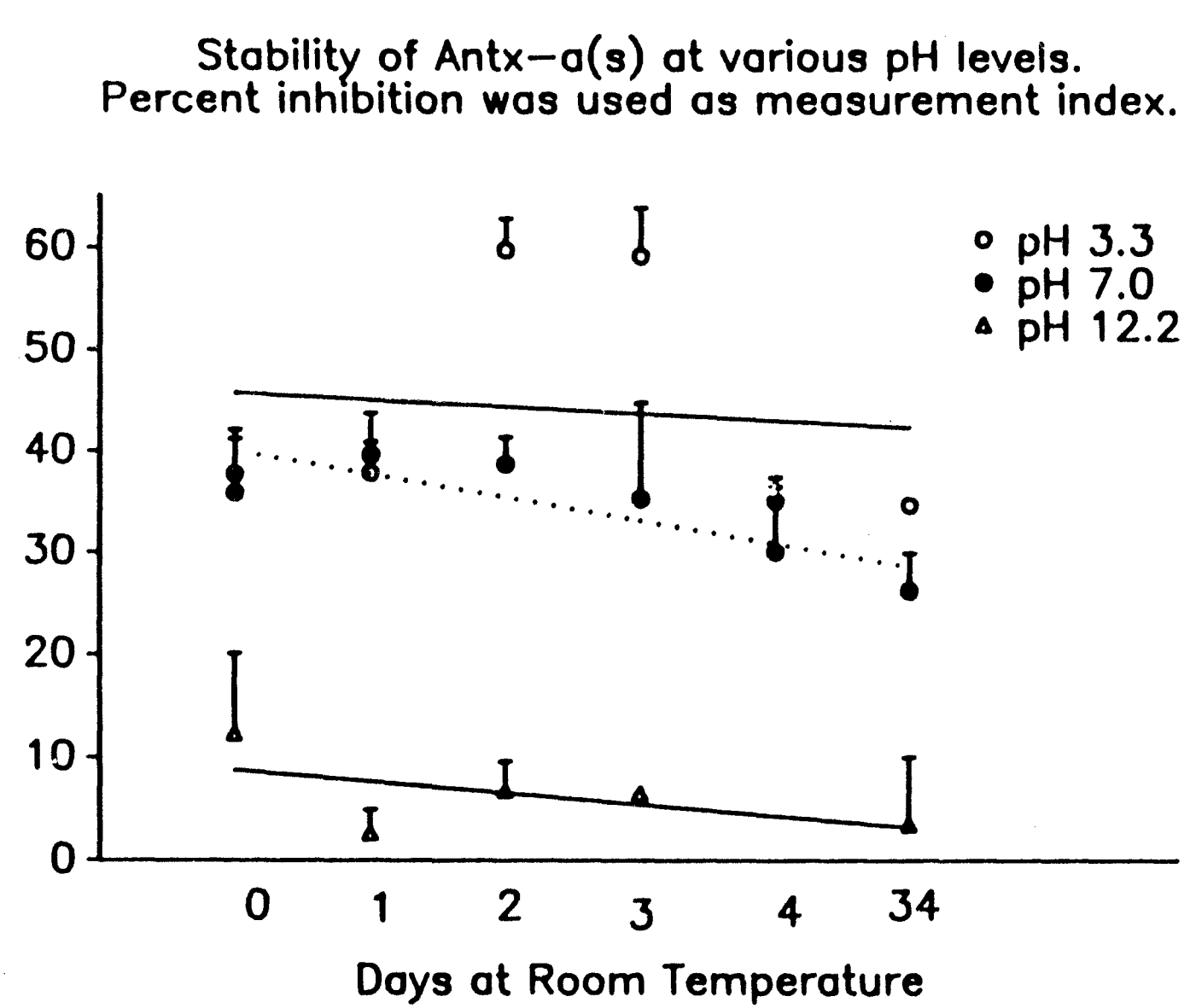


Fig. 25. ANTX-A(S) inhibition as a function of incubation pH.

7. Studies on Toxin Production in Cyanobacteria

a. Background Information:

Microcystis aeruginosa, a planktonic blue-green alga (cyanobacterium) has been reported toxic to mammals in many countries. Differences occur in the reported composition of the toxin, but in each case, the pathological symptoms have been the same (i.e. enlarged livers and short survival times).

The genetics of toxin production in Microcystis and other cyanobacterial strains have so far received little attention. From the toxic Microcystis aeruginosa colonial isolate NRC-1, Gorham (1964) isolated both toxic and nontoxic clones which indicated the genetic heterogeneity of toxin production. Vance (1977) suggested that toxin produced in Microcystis aeruginosa strain NRC-1 may be the result of lysogenic conversion of toxin strains. In Anabaena flos-aquae NRC-44-1, the toxicity was not lost after treatment with acridine orange, hence it was concluded that its toxicity was not controlled by extrachromosomal elements (Kumar and Gorham, 1975). However Hauman (1981) applied several compounds, which eliminate plasmids from other prokaryotes or select for plasmid free cells, to a South African strain of M. aeruginosa (WR70), with a consequent decrease in toxicity. These findings implicated plasmids in toxin formation by strain WR70, although plasmid visualization in extracts of this strain was not reported. Vakeria et al. (1985) reported on toxicity of strain PCC-7820 cured from its plasmids, but again the absence of plasmids had not been vigorously demonstrated. Plasmids may still be present in very low copy numbers. Furthermore it is possible that the plasmids become integrated into the chromosomal DNA (Daniell et al. 1986). Schwabe et al. (1988) reported the detection of plasmids in several toxic strains of Microcystis aeruginosa. Two strains of M. aeruginosa (HUB-5-2-4, HUB-063) harbored plasmids of 2.9 kb and 16 kb plasmids. One of the toxic strains SAG 14.85 (NRC-1) did not contain any plasmid. In contrast to this Vakeria et al. (1985) reported the presence of 3-4 plasmids in M. aeruginosa PCC-7820.

In order to understand toxin production, both quantitatively and qualitatively it is necessary to study the presence and possible role of plasmids in M. aeruginosa. The cultures grow slowly in the presence of plasmid curing agents, indicating the adverse effects of these chemical agents on algal cells. Moreover the plasmids integrate into chromosomal DNA through homologous recombination (Wilson and Morgan, 1985; Daniell et al., 1986). Exchange of genes between plasmid and host chromosome during transformation has also been well documented (Lopez et al., 1982). In studies conducted using plasmid curing experiments (Vakeria et al., 1985; Hauman, 1981; Kumar and Gorham, 1975), the plasmid may be integrated into chromosomal DNA and still the toxic polypeptide may be expressed. So further experiments are needed to confirm the role of plasmids in toxin production in M. aeruginosa which will aid in studies on growth

and toxin production aimed at stabilizing production and yields of these secondary chemicals.

b. Current Status of the Project:

Plasmid Isolation

The hepatotoxin producing strains M. aeruginosa PCC-7820, M. aeruginosa M228, Nodularia L-575 and Anabaena flos-aquae S-23-g-1-c are being used to isolate plasmids. The plasmids from the above strains were isolated using the method of Lambert and Carr (1982) with modifications as follows. Twenty-five mls of culture ($2-5 \times 10^8$ cells/ml) was centrifuged at 10,000 rpm for 10 min. The pellet was suspended in SE buffer (0.12 M NaCl and 0.05 M EDTA, pH 8.0) for 30 min. The cells were centrifuged and washed in 20 ml of lysis buffer (25 mM Tris, pH 8.0, 10 mM EDTA and 50 mM glucose). The final pellet was resuspended in 2.0 ml lysis buffer. Two ml of lysozyme solution (50 mg/ml) was added to this mixture and incubated at 37°C for one hour. Later 2.0 ml of 10% sodium laurel sarcosine was added and incubated at 50°C for 1 h. Six ml of 5 M NaCl was added and kept on ice for 2-3 hrs. The whole homogenate was centrifuged, at 17,000 x g at 4°C for 20 min., to remove chromosomal DNA and cell debris. The clear lysate was phenol extracted three times, chloroform extracted twice and the DNA was ethanol precipitated. The next day DNA was pelleted after centrifugation at 12,000 x g for 20 min., washed once with 70% ethanol and the pellet was air-dried and then resuspended in minimal volume of TE (10 mM Tns, 1 mM EDTA, pH 8.0) buffer.

Gel Electrophoresis and Visualization of DNA:

Plasmids were separated using horizontal 1% agarose gel electrophoresis, followed by straining with ethidium bromide. M. aeruginosa PCC-7820 had 2 plasmids of sizes 3 and 16 kb. whereas M. aeruginosa M228 had 2 plasmids of sizes 2.9 ad 8.0 kb. These results are consistent with the results of Schwabe et al. (1988) who found the same set of plasmids in PCC-7820 and PCC 7813 (2.6 and 16 kb) and HUB-5-2-4 and HUB-063 (2.9 and 8.5 kb). However, as yet, in Nodularia and Anabaena, we can not detect the presence of any plasmids.

In the above experiments, the cells form the stationary phase cultures were used for plasmid isolation. However these experiments will be repeated using log phase cultures, since these cultures tend to give higher copy numbers and better plasmid yields.

C. Future Work:

Purification of plasmids from agarose gel electrophoresis

The plasmids from Microcystis aeruginosa PCC-7820, and M228 will be separated on 0.8% agarose gel electrophoresis. The agarose gel piece containing the plasmid is excised and placed in

a dialysis bag with buffer. Electrophoresis causes DNA to migrate out of the gel into the dialysis bag buffer. The fragment is recovered in this buffer and purified from contaminating material using SS-phenol/chloroform extraction.

In vitro replication of plasmids:

This experiment will be done to check whether the plasmids isolated from M. aeruginosa PCC7820 and M228 will replicate autonomously in another cyanobacterial strain Synechocystis. The experiment will be done following the method of Li and Kelly (1984).

The standard replication reaction mixture contains the following in 0.1 ml: 30 mM Hepes (pH 7.5), 7 mM $MgCl_2$, 0.5 mM DTT, 100 µM (α-^{32}P) dCTP (specific activity, 3000-6000 cpm/pmol), 100 µM dATP/dGTP/dTTP, 200 µM GTP/UTP/CTP, 4 mM ATP, 40 mM phosphocreatine, 10 µg of creatine phosphokinase, 250 ng of plasmid DNA and 60 µl of cyanobacterial clear cell extract. Reactions are incubated at 37°C for various periods and then stopped by adjusting the reaction mixtures to 15 mM EDTA, 200 µg of proteinase K/ml, and 0.2% SDS. After incubation for 20-30 min at 37°C, the solution is extracted once with phenol, desalted by gel filtration on superfine Sephadex G-50 (Pharmacia), and extracted once with chloroform, and the DNA is precipitated with ethanol. The deproteinized reaction is electrophoresed through 1.5% agarose gels and then autoradiographed. The autoradiogram will give the information on the topological isomores of the plasmid templates.

Transformation of Synechocystis with plasmid DNA isolated from Microcystis aeruginosa PCC-7820 and M. aeruginosa M228.

Transformation of Synechocystis cells with plasmid DNA will be done following the method of Daniell et al. (1986). Cells or permeaplasts prepared by 2-hr treatment with lysozyme/EDTA are immediately used for transformation. To 1.0 ml of permeaplasts or cells, 1 µg of donor plasmid DNA (in 10 mM Tris/1 mM EDTA, pH 8.0 at 25°C) is added, and the suspension is incubated for different durations in sterile culture tubes on an illuminated horizontal test tube shaker at 32°C. Samples will be plated in triplicate with a series of 2-fold serial dilutions to quantify transformants.

The transformed Synechocystis cells will contain plasmids from M. aeruginosa. The plasmids will be isolated from these strains and separated using 1% agarose gel electrophoresis. The plasmid profiles will be studied to see whether the transformed Synechocystis cells harbor M. aeruginosa plasmids.

Test for toxicity:

Synechocystis, both untransformed and transformed cells will be used for mouse bio-assay. If the plasmids code for the synthesis of hepatotoxin, the transformed Synechocystis cells will be toxic to mice.

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C. SUMMARY

Toxic waterblooms of freshwater cyanobacteria are unpredictable and intermittent in occurrence. They are most often found in temperate latitudes and occur in shallow inland reservoirs, lakes, ponds, rivers, and sloughs. Cases of blue-green algae toxicosis have been verified in every continent except Antarctica. They are particularly abundant and increasingly recognized in the inland water bodies of Central/Eastern Europe, Western Asia (Ukraine), Southeast Asia/India/Japan, Southern Africa, South America and North America. An increasing number of these cases involve human contact with toxic blue-green algae, although at this time no confirmed deaths due to the toxins have been reported. Toxin groups include alkaloids, peptides and contact poisons. The alkaloids currently include anatoxin-a (a depolarizing neuromuscular blocking agent), anatoxin-a(s) (an irreversible anticholinesterase), and aphantoxin-I and II (equivalent to neosaxitoxin and saxitoxin, the major paralytic shellfish toxins). Peptide toxins are a family of cyclic hepta- and pentapeptides with similar activity. They primarily act as hepatotoxins, causing hepatocyte disaggregation and death by hemorrhagic shock. The contact toxins are at present poorly understood but current information suggests they are not related to the other blue-green toxins. All of these toxins represent potential threat agents because they are: 1) water soluble and orally toxic; 2) accumulate in high concentrations (algal blooms) making them relatively easy to collect and process into highly concentrated crude toxin preparations.

This report represents work supported by USAMRDC during the period November 1, 1987 to October 31, 1988. The contract continues to contribute directly to the establishment of a culture facility which is supplying research level quantities of known freshwater blue-green toxins. Cyclic peptide toxins are being used for basic investigations leading to an understanding of structure, function, and detection methods for these toxins. This contract supports the culture facility (which is in turn, providing material for the inhouse projects at USAMRIID) and allows further work on other freshwater blue-green algal toxins.

D. Papers Published in the Scientific Literature, and Presented at Scientific Meetings supported in part by Contract DAMD-17-87-C7019 (annual report year 1987-88).

Scientific Paper (P)/Poster (PO) Presentations
(Presenter is underlined)

P Cellular mechanisms of action for freshwater cyanobacteria (blue-green algae) toxins. Symposium - Cellular and Molecular Mode of Action of Selected Microbial Toxins in Foods and Feeds. US/Japan Coop. Program on Development and Utilization of National Resources. Chevy Chase, MD. Oct. 31-Nov. 2, 1988. (W.W. Carmichael, E. Hyde and N. Mahmood,.

P Occurrence and toxicity of toxic cyanobacteria. Symposium - Trichothecene, Blue-green Algal and Marine Toxins: Mechanism, Detection and Therapy. FASEB Summer Res. Conf. Copper Mt., CO. July 25-27, 1988. (W.W. Carmichael).

P Aquatic phycotoxins - an overview of current research directions. Nordic Symposium - Toxin Producing Algae. Oslo, Norway. Oct. 20-21, 1988. (W.W. Carmichael). Keynote address.

P Overview of cyanobacteria (blue-green algae) toxins. 9th World Congress on Animal, Plant and Microbial Toxins. Stillwater, OK. July 31-Aug. 5, 1988. (W.W. Carmichael). Plenary address.

PO Microcystin-LR induces morphologic and cytoskeletal hepatocyte changes in vitro. FASEB Summer Conf. - Trichothecene, Blue-green Algal and Marine Toxins. Copper Mt., CO. July 24-29, 1988. (S.B. Hooser, L.L. Waite, V.R. Beasley, W.W. Carmichael, M.S. Kuhlenschmidt, and W.M. Haschek).

PO Toxicity of microcystin from Microcystis aeruginosa in rats: morphologic and serum chemistry alterations. FASEB Summer Conf. - Trichothecene, Blue-green Algal and Marine Toxins. Copper Mt., CO. July 24-29, 1988. (S.B. Hooser, V.R. Beasley, W.W. Carmichael and W.M. Haschek).

PO Hemodynamics, hepatic and renal perfusion, and selected clinical pathology parameters in swine administered microcystin-LR intravenously. FASEB Summer Conf. - Trichothecene, Blue-green Algal and Marine Toxins. Copper Mt., CO. July 24-29, 1988. (R.A. Lovell, K.R. Holmes, D.J. Schaeffer, W.M. Valentine, A.M. Dahlem, W.W. Carmichael and V.R. Beasley).

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PO Reversal of cholinesterase inhibition in plasma, red blood cells and diaphragm; clinical signs, and postmortem findings in mice after intraperitoneal injection of anatoxin-a(s), paraoxon, or pyridostigmine. FASEB Summer Conf. - Trichothecene, Blue-green Algal and Marine Toxins. Copper Mt., CO. July 24-29, 1988. (W.O. Cook, A.M. Dahlem, S.B. Hooser, W.M. Haschek, K.S. Harlin, W.W. Carmichael and V.R. Beasley).

PO Regional brain cholinesterase activity in long-Evans rats injected intraperitoneally with anatoxin-a(s) compared to paraoxon. FASEB Summer Conf. - Trichothecene, Blue-green Algal and Marine Toxins. Copper Mt., CO. July 24-29, 1988. (W.O. Cook, W.W. Carmichael, and V.R. Beasley).

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PO Anatoxin-a(s) Effects on Isolated Muscle. Proc. Soc. of Toxicology - Jan. 1988 Annual Meeting, Dallas, Texas. (E.G. Hyde and W.W. Carmichael).

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Ref Rinehart, K.L., K. Harada, M. Namikoshi, C. Chen, C. Harvis, M.H.G. Munro, J.W. Blunt, P.E. Mulligan, V.R. Beasley, A.M. Dahlem, W.W. Carmichael. Nodularin, microcystin and the configuration of Adda. J. Am. Chem. Soc. 110:8557-8558.

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Ref Cook, W.O., V.R. Beasley, A.M. Dahlem, J.A. Dellinger, K.S. Harlin and W.W. Carmichael. 1988. Comparison of effects of anatoxin-a(s) and paraoxan, physostigmine and pyridostigmine on mouse brain cholinesterase activity. Toxicon 26(8): 750-753.

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Ref Cook, W.O., V.R. Beasley, S.P. Hooser, W.M. Haschek-Hock, A.M. Dahlem, K.S. Harlin, J.A. Dellinger and W.W. Carmichael. Reversibility of the inhibition of plasma, red blood cell and diaphragm cholinesterase, clinical signs, and postmortem findings in mice after intraperitoneal injection of anatoxin-a(s), compared to paraoxon and pyridostigmine. Am. J. Vet. Res. (In press).

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Ref Harada, K-I., Y. Kimura, K. Ogawa, M. Suzuki, A.M. Dahlem, V.R. Beasley, and W.W. Carmichael. Analysis and purification of naturally occurring Anatoxin-a. Toxicon. (Submitted).

E. APPENDICES

Appendix I

[1]Studies on Anatoxin-A(s) from *Anabaena flos-aquae* NRC-525-17-b-1-e: Optimized Extraction/Purification Scheme

Patti M. Thorn

Contents

1. Overview

The major focus of this research was to: (1) improve the extraction of anatoxin-a(s) from lyophilized *Anabaena flos-aquae* cells; (2) improve the subsequent purification scheme, and finally; (3) develop an alternative to the mouse bioassay for qualitative detection of anatoxin-a(s).

Enhanced extraction required the introduction of a water/chloroform and water/butanol partition scheme to facilitate pigment removal prior to toxin application on ODS (C-18) cartridges (method 2). Additionally, bulk ODS packed to a 10 ml capacity, allowed larger applications of crude toxin extract. Enhanced toxin

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[1]Supported in part by subcontract to W. Carmichael from Univ. of Illinois contract DAMD-17-85-C-5241. This contract ended on August 31, 1988.

recovery during purification was accomplished by the addition of a trituration sequence with acidified methanol and subsequently with acidified ethanol (method 2). This sequence effectively removed toxin from a methanol-insoluble precipitate which forms following drying of the eluted toxin from ODS (C-18) cartridges. TSK gel Toyopearl (with 0.05 N AcOH/MeOH) provides an efficient separation method of the toxic portion from the crude extract prior to final purification by HPLC.

In order to avoid the addition of basic salts during HPLC purification and concomitantly reduce deterioration of the alkali-sensitive anatoxin-a(s), both problems inherent to the 10 mM ammonium acetate HPLC scheme (method 1), a preferential HPLC method was developed. The method employs a preparative CN column and isocratic toxin elution with 1% AcOH mobile phase.

For the qualitative detection of anatoxin-a(s), a modified acetylcholinesterase inhibition assay was developed. This assay provides an alternative to the mouse bioassay. It is a rapid, biochemical method for location of the non-purified toxic component from a crude extract, with a detection sensitivity of less than 100 ng.

Utilization of the optimized extraction/purification scheme described in this report (method 2) has led to a four-fold increased recovery of purified anatoxin-a(s) over yields reported using method 1, and a 28-fold increased recovery of anatoxin-a(s) over yields reported in the 1986-87 annual report. Presently, an average value of 0.,29 mg toxin per gram of lyophilized cell material is obtained.

2. Isolation of Anatoxin-A(s)

A. EXTRACTION

Summaries of anatoxin-a(s) extraction/purification schemes from Anabaena flos-aquae NRC-525-17 are outlined in Figs. 1 and 2.

Method 1 represents a variation of the protocol described by Mahmood (Annual Report, 1986-87). Toxicity of lyophilized cells to be extracted is ≤ 100 mg/kg. Ten grams of lyophilized cells are routinely extracted in 25 volumes of 0.05 N AcOH/EtOH (pH 4) for 3 hrs at room temperature. The primary extraction is followed by centrifugation at 10,000 rpm for 50 min at 4°C. Extraction of the resultant pellet is repeated until toxicity is not detected using the mouse bioassay. Resultant supernatants are combined, air-dried and then reconstituted in 30 ml of acidified water (pH 4). Centrifugation at 5000 rpm for 15 min removes cellular debris prior to ODS (C-18) bond eluting. The crude toxin extract is loaded on

Figure 1. Schematic of Method 1 extraction/purification for anatoxin-a(s).

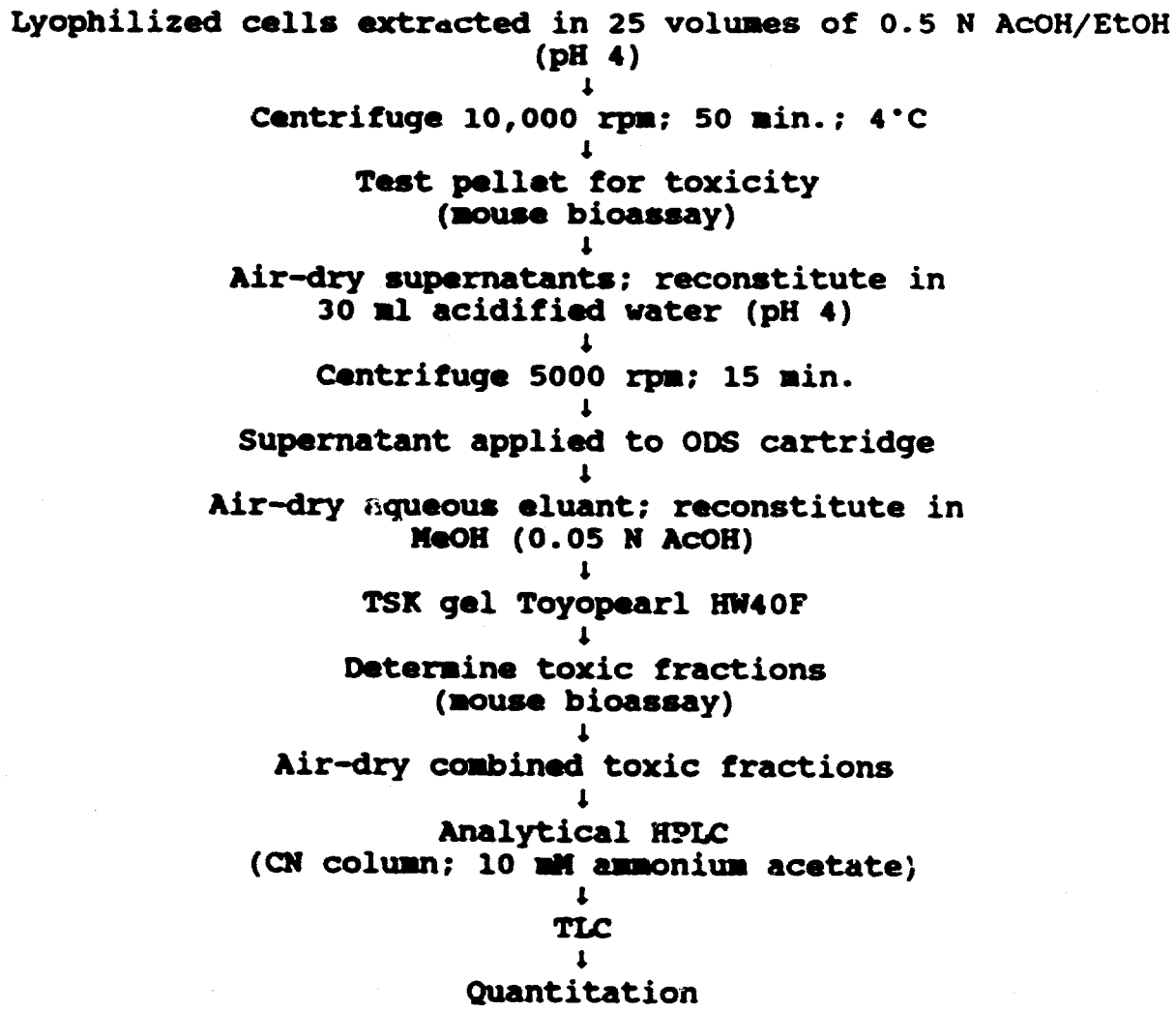
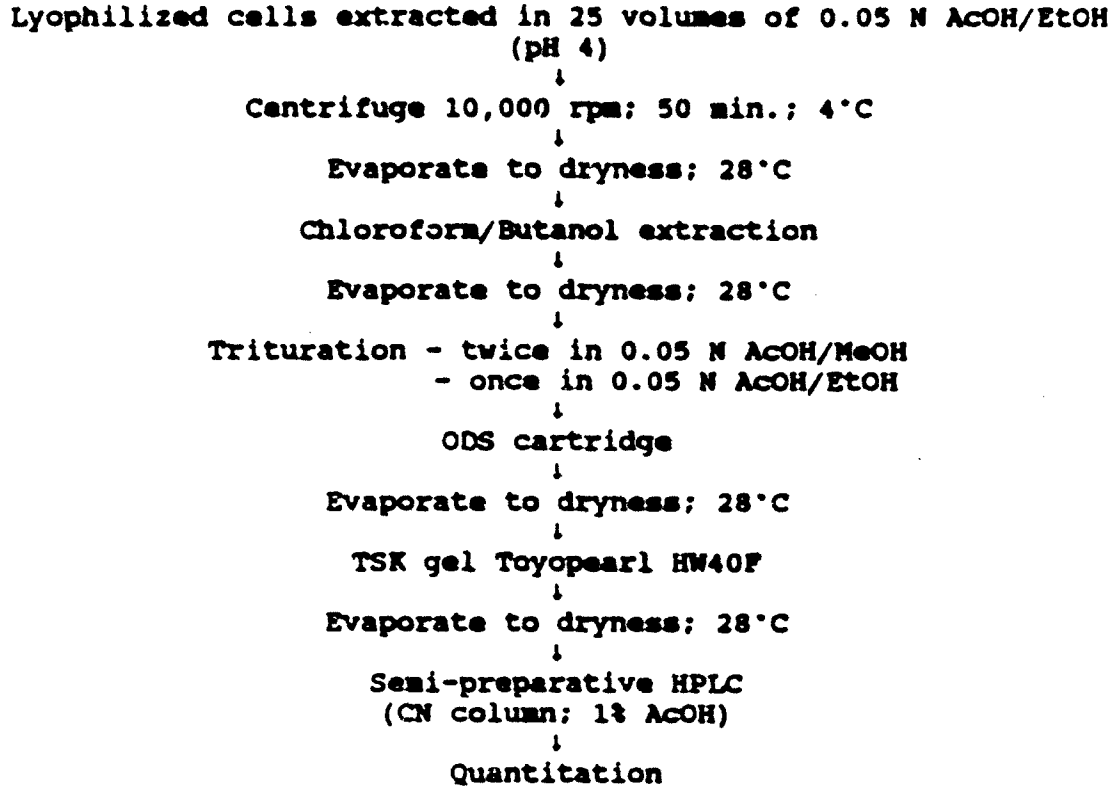


Figure 2. Schematic of Method 2 optimized extraction/purification for anatoxin-a(s).



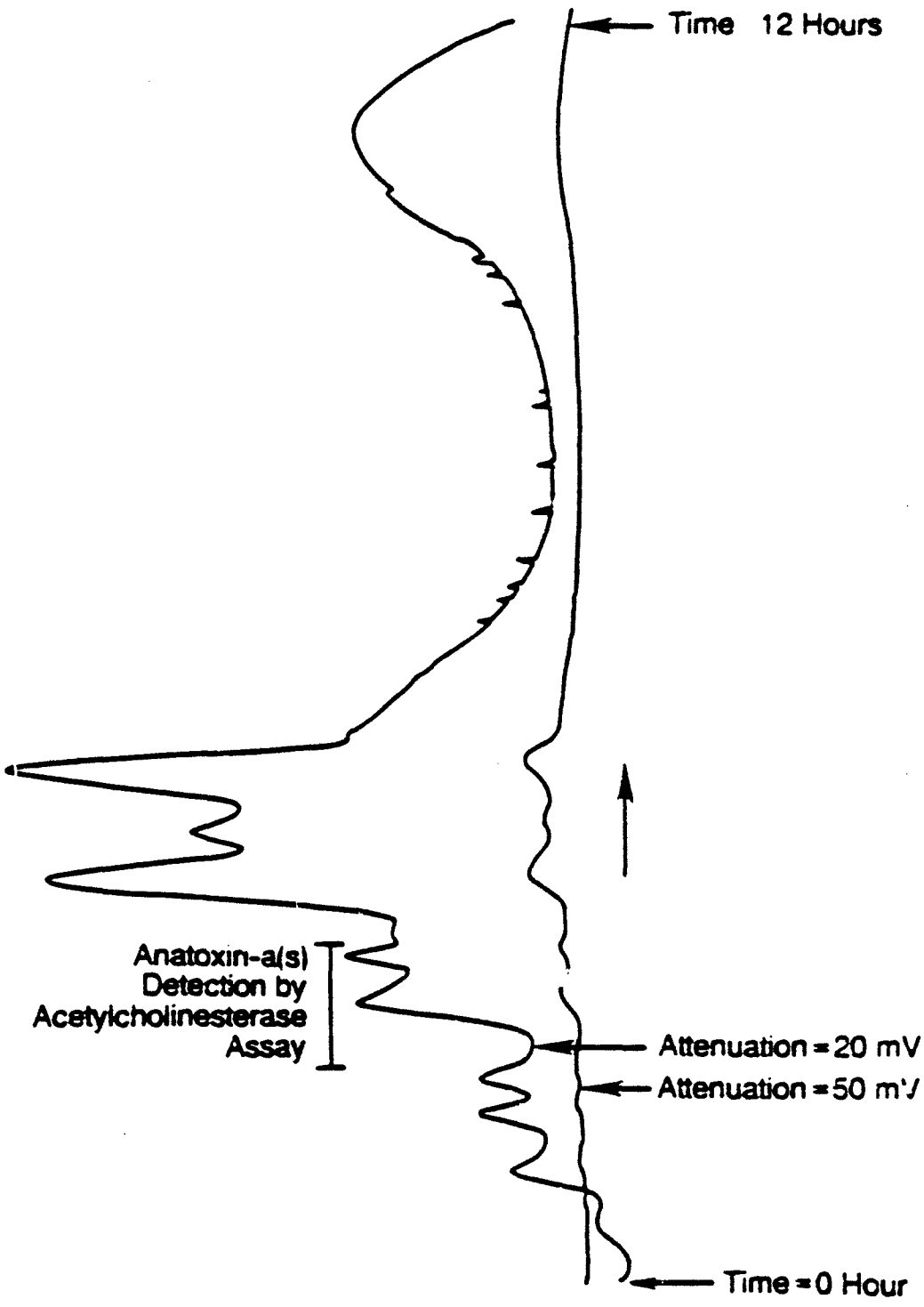
ODS cartridges (Baker SPE and Analytichem Bond Elut). The sorbent is washed with 2 ml of methanol and 5 ml of water prior to toxin extract loading. Aqueous eluant is collected, air-dried and reconstituted in 3-4 ml of 0.05 N AcOH/MeOH in preparation for TSK gel Toyopearl chromatography.

Using method 2, which represents an optimized extraction protocol, ten to thirty grams of lyophilized cells are extracted in 25 volumes of 0.05 N AcOH/EtOH (pH 4) for 3 hrs at room temperature. The primary extraction is followed by centrifugation at 10,000 rpm for 50 min at 4°C. Extraction of the resultant pellet is repeated three times in the same manner. Combined extracts are evaporated to dryness at 28°C using a Buchi 011 rotavapor. To facilitate pigment removal the extract is partitioned between equal volumes of water and chloroform. Following separation, the aqueous layer is re-extracted with an equal volume of chloroform. After separation, the chloroform layers are combined and partitioned with 20 ml of water to remove toxin (approximately 2% of total) which remains in the organic phase. The resultant aqueous phase is combined with the initial aqueous layer, and extracted twice with an equal volume of n-butanol. Combined butanol layers are then partitioned with 1% AcOH (in water) to remove toxin (approximately 4% of total) from the organic phase. Aqueous phases are combined, evaporated to dryness at 28°C and residual acetic acid is removed by azeotrophic evaporation with toluene. Due to the presence of a methanol-insoluble precipitate, the toxic fraction is triturated three times with 20 ml of 0.05 N AcOH/MeOH, and the precipitate is discarded. the extract is evaporated to dryness and further triturated with 10 ml of 0.05 N AcOH/MeOH and 10 ml of 0.05 N AcOH/EtOH. Following evaporation, the crude extract is loaded on cartridges containing 10 ml of 120 A ODS (C-18) (Yamamura Chemical Laboratories Co., LTD., Kyoto, Japan). The sorbent is washed with 10 ml of methanol and 20 ml of water prior to toxin extract loading. Following application of toxin to the cartridges, aqueous eluant is collected and evaporated to dryness. In preparation for gel chromatography, the extract is reconstituted in 8-10 ml of 0.05 N AcOH/MeOH.

B. PURIFICATION

TSK gel Toyopearl HW40F (Supelco Inc., Bellefonte, Pa.) is prepared by rinsing with water and equilibrating in 0.05 N AcOH/MeOH. The column is slurry packed to a 500 ml bed volume. The toxin extract is applied to the column which is then run as a gravity flow system. Column effluent monitored at 230 nm is collected in fractions of 6.2 ml using a Gilson fraction collector (model FC-80K). The toxic fraction generally elutes at 48-55 percent of the total column bed volume. Figure 3 represents a tracing of the Toyopearl chromatography.

Figure 3. Toyopearl HW40F chromatograph. (0.05 M AcOH/MeOH: flowrate, 30 ml/hr; 23[illegible] nm: 0.5 AUFS). Toxic fraction, as determined by the m[illegible]ified acetylcholinesterase assay, is indicated by the solid line.



C. QUALITATIVE DETECTION OF A(S)

Location of the toxic Toyopearl fraction has previously been accomplished using the mouse bioassay (method 1). A modification of the photometric Ellman assay (1), performed on Whatman filter paper rather than in a cuvette, represents an alternative method of locating the toxic fraction from a crude extract. Reagents are prepared as follows: dithiobisnitrobenzoate (DTNB), 5 mg/ml EtOH; acetylthiocholine iodide, 5 mg/ml EtOH; electric eel acetylcholinesterase, 5 units/ml KPO_4 buffer (pH 8); diisopropylfluorophosphate (DFP), 1 mg/ml EtOH; physostigmine, 1 mg/ml EtOH.

Anatoxin-a(s) extract is spotted on Whatman filter paper along with positive controls, DFP and physostigmine. DTNB and acetylcholine solutions are then sprayed on the filter paper and allowed to air dry. The enzyme solution is applied by spraying. Color development requires 20 min for optimum visualization. In a positive A(S) assay, acetylcholinesterase reacts with the Ellman substrates to yield a white concentric inhibition zone against an intensely yellow-colored background. Positive controls show a similar reaction.

D. HPLC ANALYSIS OF THE TOXIC FRACTION FOLLOWING TOYOPEARL CHROMATOGRAPHY.

Purification of anatoxin-a(s) using method 1 utilizes an analytical cyanopropyl (CN) cartridge (Altex, 4.5 x 150 mm) and isocratic elution with 10 mM ammonium acetate:water (80:20). A typical HPLC profile is shown in Figure 4. Peaks collected are checked and confirmed as toxic using the mouse bioassay.

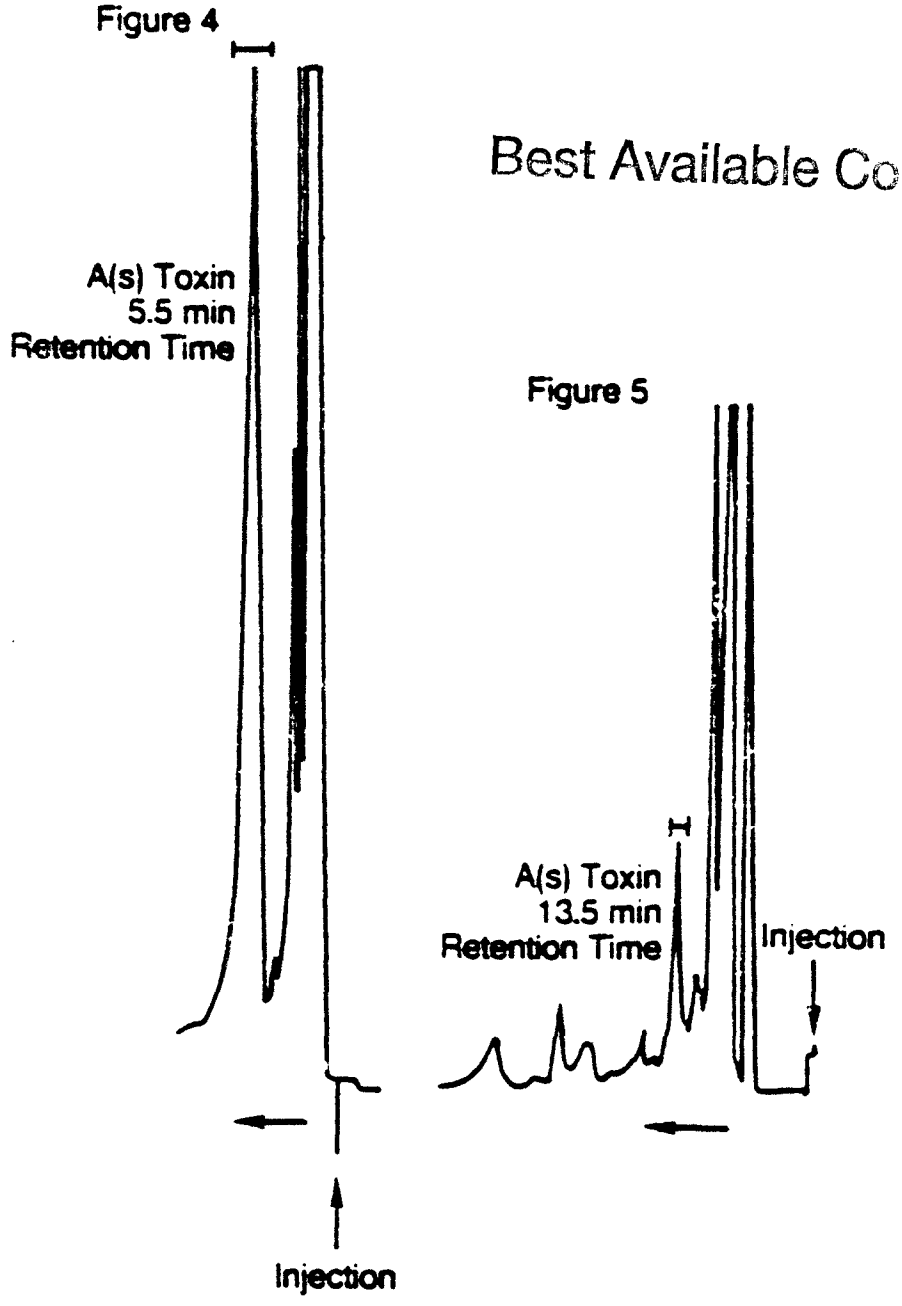
Using methods 2, a preparative CN cartridge (Alltech Econosphere, 250 x 10 mm) is used with 1% AcOH (in double distilled water). The isocratic separation is achieved on a Waters Delta Prep 3000 single pump system with valve-type solvent mixing. Detection is through the preparative cell of Waters 481 detector at 208 nm. A typical HPLC profile is shown in Figure 5, anatoxin-a(s) exhibiting a retention time of 13.5 min with a flow rate of 2 ml/min.

E. ELLMAN ASSAY FOR QUANTITATING ANATOXIN-A(S) YIELD

The photometric enzyme assay of Ellman et al. (1) is used in conjunction with the mouse bioassay to quantitate toxin yield following extraction/purification. Microliter volumes of purified A(S), reconstituted in acidified water (pH 3), are added to 0.25 units of electric eel acetylcholinesterase and incubated for 2 min. Following incubation, 3 ml of 0.1 M potassium phosphate buffer (pH 8), acetylcholine iodide (0.075 M) and dithiobisnitrobenzoate (0.01 M) are added, and the change in absorbance at 412 nm over a 15 sec interval is recorded.

Figure 4. HPLC profile of the Toyopearl toxic fraction (method 1). Altex CN cartridge, 4.5 x 150 mm; 10 mM ammonium acetate:water (80:20); flowrate, 1.5 ml/min; 230 nm; O.D. at 0.1 auf. Anatoxin-a(s) exhibits a retention time of 5 min 35 sec. Toxic peak is indicated by solid line.

Figure 5. HPLC profile of the Toyopearl toxic fraction method 2). Alltech CN cartridge. 10 x 250 mm; 1% AcOH isocratic elution; flowrate, 2.0 ml/min; 208 nm; O.D. at 0.5 auf. Anatoxin-a(s) exhibits a retention time of 13 min 30 sec. Toxic peak is indicated by dashed line.



F. ^{1}H NMR SPECTRAL ANALYSIS

^{1}H NMR spectrum of a purified A(S) sample was measured in 1% CD_3CO_2D/D_2O (Fig 5).

3. RESULTS AND DISCUSSION

Table 1 summarizes Anabaena flos-aquae lyophilized cell material extracted to date, the method used for extraction/purification and respective yields of anatoxin-a(s). Using the optimized scheme (method 2) calculated yields of toxin average 0.29 mg toxin/gram of lyophilized cell material. This represents a 4-fold increase over yields reported using method 1 (approximate yield is 0.07 mg toxin/gram lyophilized cell material) and a 28-fold increase over yields reported in the 1986-87 annual report (10 ug toxin/gram lyophilized cell material).

Location of the toxic chromatographic fractions has previously been performed exclusively by the mouse bioassay. This procedure, though effective, substantially reduces the final yield of anatoxin-a(s) due to expending the injection volume required to elicit a neurotoxic response. As an alternative detection method to the mouse bioassay, use of the photometric Ellman assay (1) scheme has not been successful. Although purified anatoxin-a(s) shows pronounced inhibitory activity against several preparations of cholinesterase (2) and is used routinely in this laboratory to quantitate purified toxin, use of the acetylcholinesterase assay as a method of qualitative toxin detection during the purification scheme has not been successful due to the interference of salts, organic solvents and pigments present in the crude extract. A modification of the Ellman assay (Matsunaga, S., personal communication) performed on Whatman filter paper with spray reagents, does represent a successful means of toxin detection from a crude extract. The presence of organic solvents, pigments or acetic acid does not affect the colorometric reaction when the filter paper is sufficiently dried. However, the presence of sufficient ammonium acetate may indicate a false positive result. In addition to utilizing less toxin for localization of toxic fractions, thus increasing the final yield of toxin, the detection sensitivity of this assay is less than 100 ng.

The HPLC purification scheme described in method 1 requires isocratic toxin elution from a CN cartridge with 10 mM ammonium acetate. Although this system provides an adequate separation of anatoxin-a(s) (Fig. 4), it was suspected that the alkali-sensitive toxin may deteriorate during subsequent lyophilization due to the presence of residual ammonia. Additionally, we have noted that lyophilized toxin weight is a poor indication of toxic activity (i.e., lyophilized weight is consistently higher than activity as determined by mouse bioassay), and that weight decreases with successive lyophilization. The latter problem was attributed to the presence of salts added via the purification scheme. The alternative HPLC purification scheme which replaces the ammonium

acetate mobile phase with 1% acetic acid, eliminates to a large degree, both of these problems.

The ^1H NMR spectrum (Fig. 6) was produced using 2.8 mg of anatoxin-a(s) extracted and purified using method 2. Though a few contaminating peaks are present (marked with arrows), this spectra is comparable to the toxic form on anatoxin-a(s) noted by other researchers (Moore, R. and S. Matsunaga, personal communication).

A total of 175.20 grams of A. flos-aquae NRC-525-17 cells were extracted and purified during the 1987-88 period with a total yield of approximately 35.7 mg. The LD_{50} (i.p.) of purified anatoxin-a(s) was determined in Swiss ICR, 15 gram mice (Table 2). Using the method of Weil et al. (3) the LD_{50} was calculated to be 25.4 ug/kg with 95% confidence limits between 20.4 and 31.7 ug/kg. All purified toxin has been utilized in structural studies, toxicological/physiological studies.

Figure 6. ^{1}H NMR spectra of a purified A(s) toxin sample. x = impurities.

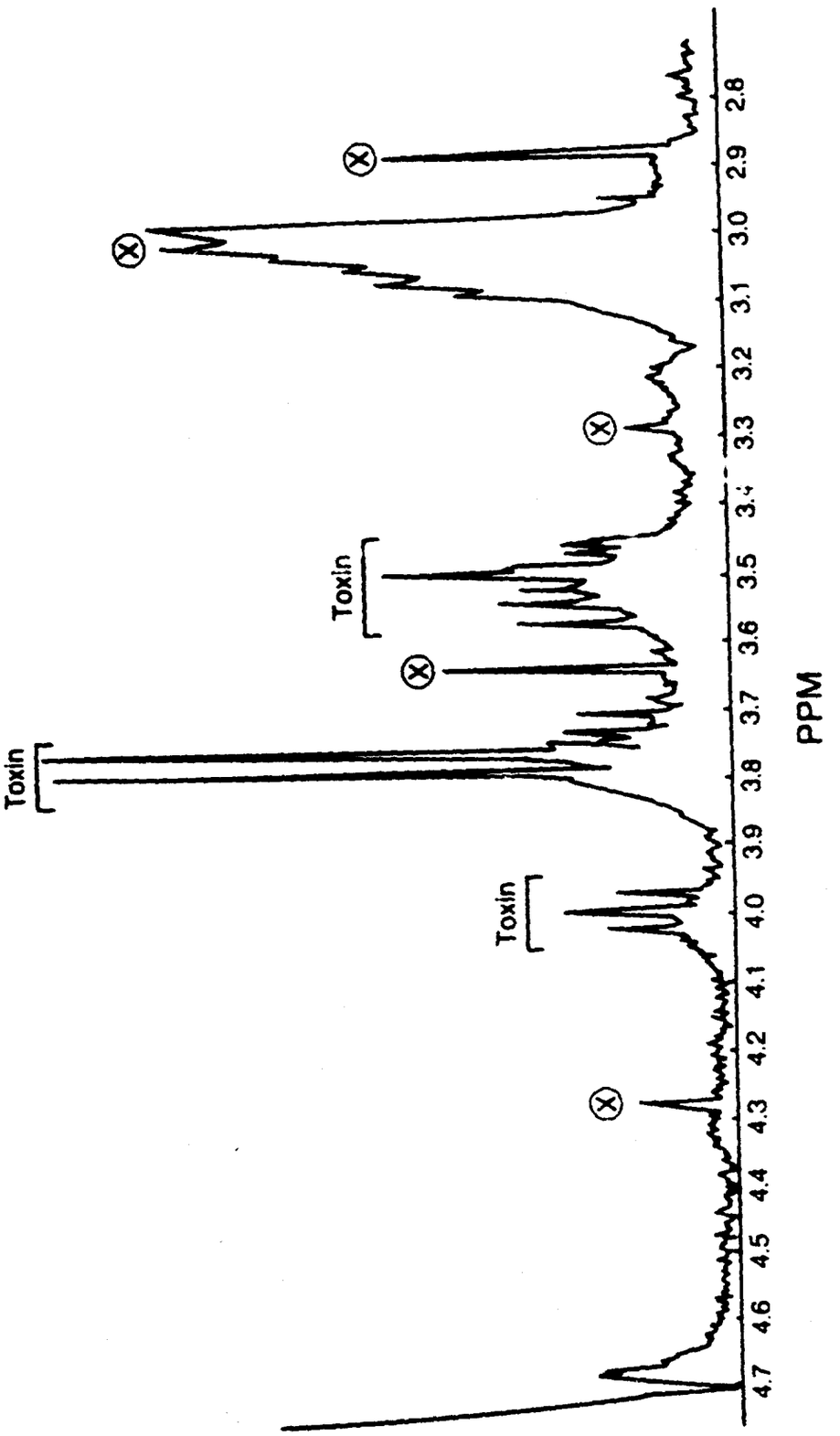


Table 1. Yield of Anatoxin-A(s)

Extraction #	Cells Extracted (g)	Extraction/ Purification Method	Wt (mg)	Toxin Yield Toxicity (MU)[A]	Ellman (mg)
1	9.60	1	0.40	-	-
2	4.60	1	1.10	20	-
3	11.91	1	-	940	1.26
4	5.00	1	0.80	-	-
5	**	1	31.90	685	0.71
6M[C]	27.40	2	2.80	-	-
7	10.12	1	2.50	1500	95.20
7		2	* 1.00	1820	1.35
8	11.43	1	1.30	150	48.20
8		2	* 0.10	180	0.17
9	11.23	1	8.40	-	-
9		2	* 4.00	2300	2.76
10	10.27	2	2.90	3670	2.53
11	**	2	0.40	630	0.38
12	18.89	2	-	-	-
13	27.64	2	9.00	7000	6.96
14	16.97	2	-	-	-
15M[C]	31.40	2	7.00	-	-
16M[C]	14.60	2	8.00	-	-

A 1 Mouse Unit = 1 ug

B Weight not determined. Sample comprised of miscellaneous semi-purified extracts.

C Extraction/Purification performed by S. Matsunaga at W.S.U. laboratory.

* Lyophilized A(s) was reconstituted in 1% AcOH and run on HPLC using 1% AcOH method.

Table 2.

Dose (i.p.) ug/kg	# animals treated	Death (24 hr limit)	Survival Time +/- S.E. (min.)
5	5	0	-
10	5	0	-
15	5	0	-
20	5	1	55.4
30	5	4	14.3 ± 3.5
40	5	5	10.8 ± 1.3

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3. Weil, C., C.P. Carpenter and H.F. Smyth (1953). Am. Ind. Hygiene Ass. Quart.

APPENDIX II

Schedule[1] of deliverables supported on contract DAMD17-87-C-7019 and on a subcontract from contract DAMD17-85-C-5241 (Univ. of Illinois--V.R. Beasley), for the time period November 1, 1987-October 31, 1988.

Date Sent*	Description	Amount (mg)	Receiver	Comments
11/87	Nodularin	24.0	K.L. Rinehart	Univ. of Illinois
12/87	Microcystin-LR	159.9	D.L. Bunner	USAMRIID
12/87	Microcystin-LR	19.4	A.M. Dahlem	Univ. of Illinois
12/87	Microcystin-(?)	1.5	K. Mereish	USAMRIID (for side peak analysis)
12/87	Microcystin-(?)	9.0	K. Mereish	USAMRIID (for side peak analysis)
1/88	Anatoxin-a(s)	0.3	H. Hines	USAMRIID
3/88	Anatoxin-a(s)	1.0	R.E. Moore	Univ. of Hawaii
3/88	Microcystin-LR	75.2	D.L. Bunner	USAMRIID
3/88	Microcystin-LR	9.1	V. Beasley	Univ. of Illinois
3/88	Microcystin-LR	4.8	D. Morton	Frostburg State Univ.
3/88	Anatoxin-a(s)	0.6	E. Hyde	Wright State Univ.
5/88	Anatoxin-a(s)	0.8	E. Hyde	Wright State Univ.
6/88	Anatoxin-a(s)	1.4	E. Hyde	Wright State Univ.
7/88	Anatoxin-a(s)	1.0	H. Hines	USAMRIID
7/88	Anatoxin-a(s)	17.8	R.E. Moore	Univ. of Hawaii
7/88	Anatoxin-a(s)	2.3	R.E. Moore	Univ. of Hawaii
8/88	Nodularin	18.1	D.L. Bunner	USAMRIID
9/88	Anatoxin-a(s)	4.0	D.L. Bunner	USAMRIID
9/88	Nodularin	19.6	D.L. Bunner	USAMRIID
10/88	Microcystin-YR	1.7	D.L. Bunner	USAMRIID
10/88	Anatoxin-a(s)	2.0	V. Beasley	Univ. of Illinois
10/88	Anatoxin-a(s)	2.0	E. Hyde	Wright State Univ.
10/88	Anatoxin-a(s)	0.8	V. Beasley	Univ. of Illinois
10/88	Microcystin-LR	108.0	D.L. Bunner	USAMRIID
10/88	Anatoxin-a(s)	3.0	D.L. Bunner	USAMRIID

* All shipments to USAMRIID wrere Federal or UPS Express

[1] Additional details .n these deliverables can be found on p. 32-35; 93, 95.

APPENDIX III

LETTER TO THE EDITOR

TOXICON

1988, Vol. 26(11):971-973

TOXICON Vol. 26 (11):971-973

LETTER TO THE EDITOR

Naming of Cyclic Heptapeptide Toxins of Cyanobacteria (Blue-green algae)

In 1878 George Francis published the first written report of animal poisoning by a cyanobacterium (blue-green alga) (Francis 1878). However, it has only been in the last 30 years that a significant amount of information has been published on both the structure and function of the neurotoxic alkaloids and hepatotoxic peptides of cyanobacteria. The neurotoxins are referred to as anatoxins (Carmichael and Gorham 1978) while the hepatotoxins have been called Fast-Death Factor (Bishop et al. 1959), Microcystin (Konst et al. 1965), Cyanoginosin (Botes et al. 1984), Cyanoviridin (Kusumi et al. 1987) and Cyanogenosin (apparently a misspelling of cyanoginosin) (Painuly et al. 1988).

Since 1965, microcystin is the term most frequently used when describing cyclic peptide hepatotoxins produced by strains or blooms of Microcystis. Cyanoginosin-"XY" is the term that has been applied to chemically defined monocyclic heptapeptide hepatotoxins isolated from strains of Microcystis aeruginosa. The derivation of the term is "cyano" from cyanobacteria and "ginosin" from aeruginosa. The most useful aspect of this terminology results not from the term cyanoginosin but from the two letter suffixes "XY" which designate the two variant "L" amino acids found in all of the cyclic heptapeptide hepatotoxins examined to date. These "L" amino acids have also proven to be the essential variants between toxins

isolated from a particular strain of M. aeruginosa (Carmichael 1986).

General Structure for the Hepatotoxic Heptapeptides

$$\text{Cyclo}(\text{-D-Ala}^{1}\text{-L-"X"}^{2}\text{-D-erythro-}\beta\text{-methyl-Asp}^{3}\text{-L-"Y"}^{4}\text{-ADDA}^{5}\text{-D-Glu}^{6}\text{-N-Methyldehydro-Ala}^{7})$$

X = Leucine (L), Arginine (R), Tyrosine (Y)

Y = Arginine (R), Alanine (A), Methionine (M)

"XY" combinations for heptapeptide toxins currently defined: LR; LA; YA, YM, YR, RR

ADDA = 3-amino-9-methoxy-2,6,8-trimethyl-10-pheryldeca-4,6-dienoic acid

It is now known, however, that other species of Microcystis (i.e., M. viridis (cyanoviridin)) and genera not within the same order (i.e., Anabaena and Oscillatoria) also produce cyclic heptapeptides that fit the above generalized structure (Kusumi et al. 1987; Krishnamurthy et al. 1986a,b; Eriksson et al. 1987). It is also now known that cyanoviridin, which was the RR heptapeptide variant, is present in M. aeruginosa (Watanabe et al. 1988) and as a desmethyl heptapeptide in Oscillatoria agardhii var. and var. isothrix (Krishnamurthy et al. 1986b).

In view of this long acceptance and use of the term microcystin in the medical and veterinary literature, and the recent identification of the cyclic heptapeptides among other species of Microcystis and other cyanobacteria genera, it seems more appropriate to retain the association with the genus. We would,

therefore, like to propose that the original term "microcystin" (MCYST) plus the suffix "XY" (designating the variant L-amino acids) be recognized as the basis for naming all existing and future monocyclic heptapeptide hepatotoxins of cyanobacteria. A summary of this reasoning follows: 1) Microcystin has been used since the 1960's to refer to peptide hepatotoxins of cyanobacteria - especially those of Microcystis. 2) Microcystin can also designate toxins from other species of the genus Microcystis or from other genera in which toxins are now being found (Kfir et al. 1986, Eloff 1987, Gathercole and Thiel 1987). 3) The sequence of the letters for the "L" amino acid suffix should follow that used by Botes (1984) in that the first letter should designate the amino acid closest to the D-alanine position (i.e. microcystin-LR). Only two exceptions within the five invariant "D" amino acids have been reported to date. These are D-aspartic acid in place of methyl aspartic acid and alanine in place of N-methyldehydroalanine (Krishnamurthy et al. 1986b). Such variations can be named by a prefix to microcystin and numbering the amino acids affected. This will result in the term "desmethyl 3-" and "didesmethyl 3,7-" respectively to describe these two known variants of the "D" amino acids. Cyclic peptide toxins with fewer or greater than seven peptides or peptide-linked components should be named according to

the genus from which they are first isolated or to their chemical composition relative to the known microcystins.

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